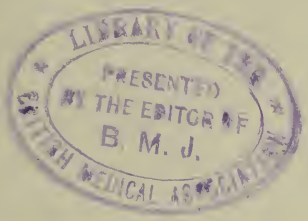

A MANUAL OF SELECTED BIOCHEMICAL METHODS

AS APPLIED TO

Urine, Blood and Gastric Analysis

BY

FRANK P. UNDERHILL, Ph.D.

Professor of Pharmacology and Toxicology,
School of Medicine, Yale University

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PREFACE

This manual is the outgrowth of a course in biochemical methods given by the undersigned in the Yale School of Medicine. Requests for copies of the mimeographed manual have been so numerous that it has seemed desirable to print it in book form. With this point in view several methods not originally in the course have been included in order to give the manual a more general application. It is believed that the methods herein described will meet not only the needs of medical schools desiring a fundamental course in biochemical methods, but will also be useful for physicians, research and commercial laboratories, and hospitals.

No claim of originality is made for any of the methods. They have been compiled from such sources as the Journal of Biological Chemistry, the American Journal of Physiology, Journal of Physiology, etc., various text books, such as Hawk's Practical Physiological Chemistry, Macleod's Physiology and Biochemistry in Modern Medicine, Folin's Manual of Biological Chemistry, Treadwell-Hall Analytical Chemistry, Fresenius-Cohn Quantitative Chemical Analysis, etc.

To Dr. Michael Ringer I am indebted for aid in selecting and testing methods and to Miss M. Eloise Montague my thanks are due for preparation of the manuscript and proof reading.

Frank P. Underhill.

New Haven,
July 1, 1921.

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TO THE STUDENT

To be of value laboratory work must be carried through with a complete understanding of what one is attempting to accomplish. Without such a conception the work is of little more value to the student than any other form of mechanical exercise.

A student should never begin a particular experiment until the complete directions have been read over and an understanding gained of the problem and the way in which it is to be carried through. If there are any points which you do not understand consult the instructor. Be certain that you have all the apparatus and solutions essential before beginning work. Do not begin an experiment unless there is sufficient time to carry it to a point where it may be left without detriment to the result.

At times it may happen that the results obtained will be different than were anticipated. If so, consult the instructor. It may be that some error in technique has been made which can be easily remedied by the instructor. Or again, the results may be correct and your expectations wrong. At any rate you should not feel any hesitancy about reporting results which do not appear to you to be correct. One should not feel it any disgrace to fail to obtain an accurate result at the first trial with a new quantitative method. In fact skilled chemists often fail at first. By repetition of the work skill in technique is obtained. You should remember that it is better to do one thing well, to learn one method accurately, than to carry through a dozen things in an inaccurate manner. "Remember that you are working for your own benefit and that honesty is the first requisite of success in all walks of life, but above all it is the foundation stone of science."

GENERAL DIRECTIONS OF WORK

FOREWORD

NEATNESS and order are the first requisites of proper laboratory work of any kind. Neat workers only can be accurate manipulators. Orderly work leads to fewer mistakes than work which is done hurriedly and without system or plan.

In order to obtain proper quantitative results absolute cleanliness is essential. All of the apparatus used should be left in a chemically clean condition. Most of the apparatus can best be cleaned by rinsing well with warm water, then washing with soap and water and brush or cloth, then again rinsing well with warm water, and finally rinsing with distilled water. Never dry beakers or other chemical vessels with a towel. If it is necessary to dry a vessel quickly rinse with alcohol and ether and dry with a current of air or dry by gently warming after rinsing with distilled water. Use judgment in cleaning your apparatus. Always use the appropriate solvent first, then remove the excess of the solvent and continue the washing as above. Pipettes and burettes after the preliminary cleaning with the appropriate solvent and rinsing as above indicated, often require further treatment with cleaning mixture. Finally rinse well with warm water and distilled water. Do not use cleaning mixture unless it is necessary.

In measuring reagents do not insert the pipette into the reagent bottle, but pour off about the amount desired into a dry vessel and then measure therefrom by the pipette. *Never return solid or liquid reagents to the stock bottles.*

Caution

Acetone, alcohol, ether, benzine, glacial acetic acid, and toluene are inflammable. Never heat any of them over the free

flame. Heat them on the steam, water, or electric bath. Have no lights near when pouring ether, acetone, benzine, or toluene from one vessel to another. In case of fire, if in a dish or flask, cover it with a wet towel, by doing this you shut out oxygen, which is necessary for the maintenance of the fire. Water may be added to an alcohol or acetone fire.

LABORATORY MANUAL

PART I

GENERAL METHODS

MEASURING INSTRUMENTS

In volumetric analysis the instruments most often employed are burettes, pipettes, measuring flasks, and measuring cylinders.

Burettes are tubes of uniform bore throughout the whole length; they are divided into cubic centimeters; they are closed at the bottom by means of a glass stopcock or by a piece of rubber tubing containing a glass bead. The latter form is used as follows: The tubing is seized between the thumb and forefinger at the site of the glass bead; by means of a gentle pressure a canal is formed at one side of the bead through which the liquid will run out. Instead of the glass bead an ordinary pinchcock is frequently used.

Pipettes. A distinction must be made between the full pipette and the measuring pipette. The full pipette has only one mark upon it, and serves for measuring off a definite amount of liquid. They are constructed in different forms; usually they consist of a glass tube with a cylindrical widening at the middle; the lower end is drawn out to a fine opening. Measuring pipettes are burette-shaped tubes graduated into cubic centimeters and drawn out at the lower end as with the full pipettes.

Measuring flasks are flat-bottom flasks with narrow necks provided with a mark, so that when they are filled to this point they will contain a specified volume. They serve for the preparation of standard solutions and for the dilution of liquids to a definite volume.

Measuring cylinders are graduated into cubic centimeters and are used only for rough measurements.

In order that accurate results may be obtained all instruments used should be correctly calibrated. It should never be taken for granted that a particular instrument is correct, but it should always be subjected to a careful test. Ascertain from the instructor whether the burettes, etc., have been calibrated. For methods of calibration see below. Be certain that you understand how to read burettes and to measure accurately volumes of fluid with pipette, volumetric flask, and measuring cylinder.

For the gravimetric methods which you are to learn it will be essential that you receive practice in the use of the delicate balances. This instruction may be received at periods to be determined by your instructor.

CALIBRATION OF VOLUMETRIC APPARATUS [1]

All apparatus used for accurate work must be calibrated. Except for that checked by the Bureau of Standards no commercial apparatus is entirely reliable, errors exceeding 1 per cent being frequent.

Temperature C°.	Weght of 1 c.c. of of water in gm.	Voume of 1 gm. of water in c.c.
15	0.9981	1.0019
16	0.9979	1.0021
17	0.9977	1.0023
18	0.9976	1.0024
19	0.9974	1.0026
20	0.9972	1.0028
21	0.9970	1.0030
22	0.9967	1.0033
23	0.9965	1.0035
24	0.9963	1.0037
25	0.9960	1.0040
26	0.9958	1.0042
27	0.9955	1.0045
28	0.9952	1.0048
29	0.9949	1.0051

[1] Medical War Manual, No. 6, 1918, p. 71.

Flasks are calibrated by weighing into them the amount of water necessary to make the desired volume at the temperature of calibration. The following table shows the weights of water over the range of ordinary room temperature which fill a volume of 1 c.c. The figures are corrected for the weights of air displaced by the water and by the brass weights. The water should be weighed to 1 part per 1000, i.e., the water held by a 10 c.c. flask is weighed to 0.010 gm., but a liter flask is sufficiently accurate if within 1 gm.

Burettes are calibrated by allowing them to deliver distilled water, 2 c.c. at a time, into a bottle and weighing the water. The bottle should contain a layer of paraffin oil a few millimeters thick. This floats on top of the water and prevents loss by evaporation. It is not necessary, therefore, to stopper the bottle. The grams of water noted are multiplied by the volume of 1 gm. at the temperature observed. If the results do not agree to within 0.05 c.c. (for a 25 to 50 c.c. burette) with the readings the corrections should be plotted on a sheet of coördinate paper, which is hung by the burette for reference.

The following figures for the first 10 c.c. of a burette serve as an example:

Burette reading, c.c.	Weight of water delivered at 22° C. gms.	Volume of water delivered (=wt.×1.0033) c.c.	Corrections to burette, c.c.
2	2.000	2.006	0.01
4	4.002	4.008	0.01
6	6.009	6.017	0.02
8	8.020	8.050	0.05
10	10.020	10.050	0.05

Pipettes are calibrated by filling to the mark with distilled water and discharging into a weighing bottle. The water delivered should be weighed to within 1 part per 1000. If the mark is not accurate a correct one should be made with a wax pencil, subsequently etched in and indicated by an arrow.

Pipettes may be calibrated for either *drainage* or *blow-out* delivery. For drainage the tip of the pipette is allowed to touch the side of the receiving vessel as delivery is finished and a drop of

liquid remains in the tip. For blow-out delivery this final drop is expelled. The expulsion is conveniently effected by closing the upper end of the pipette with the right forefinger and warming the bulb by gripping it with the left palm. The expansion of air in the bulb forces the last drop of water out of the tip. For all pipettes below 5 c.c. blow-out delivery should be used. Unless all of the pipettes in the laboratory are calibrated for either blow-out or drainage delivery each pipette must be etched " Blow-out " or " Drainage."

USE OF PYCNOMETERS[1]

The volume held by a pycnometer is determined by weighing the water which it contains and multiplying this weight in grams by the volume of 1 gram of water at the observed temperature (i.e., by 1.0028 if temperature is 20°). The density of any liquid is determined by weighing the pycnometer full of the liquid and dividing by the volume. The density is thus determined in absolute units, the density of water at 4° being taken as 1.

Urine specific gravities usually refer to water at the same temperature (rather than water at 4°) as unity. In this case the weight of urine held by the pycnometer is divided by the weight of water held at the same temperature. Such a specific gravity, if taken at 25°, for example, is indicated by $D_{\frac{2}{2}}^{\frac{5}{5}}$. If water at 4° is taken as the unit, as is done in tables of densities of acids, alcohol, etc., the density is indicated by $D_{4}^{\frac{25}{}}$.

Any vessel into which a volume of liquid may be accurately measured may serve as a pycnometer. A pipette calibrated to *contain* 1, 2 or more cubic centimeters may be used

USE OF THE BALANCE[2]

See that the balance is perfectly level, as indicated by the plumb-bob or spirit-level; if it is not, make it so by turning the adjusting screws at the right and left front corners.

Always place the object to be weighed on the left-hand pan, and the weights on the right-hand pan. One reason for this is to equalize errors through possible inequalities in the length of the

[1] Medical War Manual, No. 6, 1918, p. 73.
[2] Ibid., p. 68.

two arms of the beam. To obtain the true weight of a substance when extreme accuracy is required, counterbalance it with sand or weights and then replace the object with weights; or weigh in one pan and then exchange the substance and weights, weigh again, and take the mean of the weights so obtained.

The beam and pans must always be supported before adding or removing weights, and the weights must be handled only with forceps.

To avoid errors in noting weights always count them twice: (1) by noting those missing from the box; (2) by noting the weights as they are taken from the pan and replaced in the box.

All objects must be at room temperature when weighed. Warm objects cannot be weighed accurately as currents of air are caused which introduce an error.

Crucibles should be cooled in a desiccator when the precipitates weighed in them take up water from the air. If the crucible gains weight measurably during the weighing it should be reheated and weighed again very quickly. The weights to balance the crucible approximately are in this case placed on the pan before the crucible is removed from the desiccator.

A platinum crucible should remain in the desiccator ten to fifteen minutes and a porcelain crucible twenty to twenty-five minutes before weighing.

When, as in the case of barium sulphate, the precipitate is not hygroscopic a desiccator should not be used. Accurate results are more readily obtained when the crucible, both before and after the precipitate is in it, can be cooled in the open. The time required for cooling is about half as long as in a desiccator.

The supports of beam and pans must be lowered gently to avoid injury to the knife edges.

The balance case must never be left open or with the beam unsupported, and the rider must be removed from the beam. When not in use the balance should be protected from fumes and dust by the regular use of a rubber covering placed over the case.

Be careful to avoid spilling the substance to be weighed on the pans or on the floor of the balance case. If this happens, remove at once by dusting carefully with a camel's-hair brush.

Liquids must be weighed in closed weighing bottles, and solids in weighing bottles, watch-glasses, or aluminum pans.

From time to time determine the true zero point of the scale

by noting the point at which the rider must be placed to make the empty pans balance.

The point of balance in weighing is always determined by adjusting the weights until the pointer swings equal distances to right and left of zero on the scale. It is never determined by adjusting the loads until the pointer remains stationary when released.

INDICATORS [1]

In order to determine the amount of acid present in an unknown solution an alkaline solution of known strength is required; and conversely, in the analysis of a base an acid solution of known strength is required. In both cases the " end-point " of the reaction is determined with the aid of a suitable indicator.

The accuracy of the results depends largely upon the choice of the indicator. All indicators employed for the titration of acids and alkalies are either acids or bases. They behave like weak acids and bases whose ions and unionized molecules have different colors. The color change is due probably to a rearrangement of the molecule to a colored form. This rearrangement is probably due to the dissociation of the molecule, the undissociated molecule not rearranging. Since the indicators are acids of different avidities, that is, since they have different amounts of dissociation, some are weaker than others. Accordingly some are able to form salts in sufficient amounts to give a perceptible color in the presence of more acid than are others which are weaker.

A neutral solution is one in which there are equal numbers of hydrogen and hydroxyl ions. An acid solution has a preponderance of hydrogen ion and an alkaline solution an excess of hydroxyl ion. All indicators do not show changes in color at the true neutral point, but at some fixed figure of acidity or alkalinity, that is at a definite hydrogen or hydroxyl ion concentration. Indicators which change color at the true neutral point are litmus and rosolic acid, while phenolphthalein changes color in a slightly alkaline solution. Congo red, sodium alizarin sulphonate and tropaeolin 00 are examples of indicators which change color in an acid solution.

Organic acids in general are not sufficiently strong, i.e., do not dissociate with the production of enough hydrogen ion, to cause

[1] Modified from Hawk: Practical Physiological Chemistry, Sixth Edition, 1918.

color changes in dilute solutions with indicators of the last-mentioned class. Litmus, rosolic acid, and phenolphthalein, however, change at so low a hydrogen ion concentration that they are affected by dilute solutions of organic acids and may be used for their titration. Even very dilute solutions of *mineral* acids are sufficiently acid to produce color changes with Congo red, alizarin, etc., and hence these indicators may be used in the titration of mineral acid. Phenolphthalein which changes color in a weakly alkaline solution indicates the presence of acid combined with weakly alkaline substances (as protein) as well as other types of acid such as acid salts, and, hence, is used in the titration of solutions for their *total* acidity.

The hydrogen ion concentration of pure water or a neutral solution is approximately 1×10^{-7}, being expressed as approximate moles of hydrogen ion per liter. That is, water is a 1/10,000,000 N solution of hydrogen ions. The concentration of hydroxyl ions in pure water or a neutral solution is exactly equal to that of the hydrogen ions, so that water may be considered to be an N/10,000,000 alkali as well as an N/10,000,000 acid. Hydrogen ion concentrations are often expressed for the sake of brevity as their logarithms with the sign reversed. For example the logarithm of 1×10^{-7} would be -7.0 and according to this notation the H ion concentration would be expressed as $P_H=7.0$. The product of the hydrogen ion concentration (H^+) by the hydroxyl ion concentration (OH^-) is constant at about 1×10^{-14} so that as (H^+) increases from 1×10^{-7} ($P_H=7.0$) to 1×10^{-4} ($P_H=4.0$) the (OH^-) falls to 1×10^{-10}, and *vice versa*. It must be borne in mind that higher figures for the logarithmic notation indicate lower figures for (H^+). The hydrogen ion concentration at which certain indicators commonly used in titration work change color, are indicated below.

Tests with Indicators.—Prepare a series of solutions of varying acidities as outlined below. Introduce 5 or 10 c.c. portions of each of these into a series of test-tubes and add to each a few drops of a solution of Tropaeolin 00. Make a note of the colors produced, in the spaces left for this purpose. In the same way test out the other indicators mentioned, in order, using in each case a few drops of the indicator solution. The tests using the last three mentioned indicators, Günzberg's, Boas' and Tropaeolin (evaporation test), are carried out differently, as indicated below.

Indicator.	Hydrogen Ion Concentration.	True Nature of Solution when the Color Changes.
Phenolphthalein	Between 1×10^{-8} and 1×10^{-9}	Alkaline
Neutral red	1×10^{-7}	Neutral
Rosolic acid	1×10^{-7}	Neutral
Litmus	Between 1×10^{-6} and 1×10^{-7}	Neutral
Sodium alizarin sulphonate	Between 1×10^{-5} and 1×10^{-6}	Acid
Congo red	Between 1×10^{-5} and 1×10^{-6}	Acid
Dimethyl-amino-azobenzene	Between 1×10^{-3} and 1×10^{-4}	Acid
Methyl orange	Between 1×10^{-2} and 1×10^{-3}	Acid
Tropaeolin 00	1×10^{-2}	Acid

Special Tests for Free HCl.—Perform the following tests on the solutions as outlined above and tabulate the results.

1. *Günzberg's Reagent.*[1]—Place 1 to 2 drops of the reagent in a small porcelain evaporating dish and carefully evaporate to dryness over a low flame. Insert a glass stirring rod into the mixture to be tested and draw the moist end of the rod through the dried reagent. Warm again gently and note the production of a purplish-red color in the presence of free hydrochloric acid.

2. *Boas' Reagent.*[2]—Perform this test in the same manner as 1, above. Free hydrochloric acid is indicated by the production of a rose-red color which becomes less pronounced on cooling.

3. *Tropaeolin* 00.[3]

$$(C_6H_5)NH-C_6H_4-N=N-C_6H_4-SO_3Na.$$

Place 2 drops of the solution to be tested and 1 drop of the indicator in an evaporating dish and evaporate to dryness over a low flame. The formation of a reddish-violet color indicates free hydrochloric acid.

This test may also be conducted in the same manner as 1, above.

[1] Günzberg's reagent is prepared by dissolving 2 gms. of phloroglucinol and 1 gm. of vanillin in 100 c.c. of 95 per cent alcohol.

[2] Boas' reagent is prepared by dissolving 5 gms. of resorcinol and 3 gms. of sucrose in 100 c.c. of 50 per cent alcohol.

[3] Prepared by dissolving 0.05 gm. of tropaeolin 00 in 100 c.c. of 50 per cent alcohol.

TABULATION ON RESULTS OF TESTS ON INDICATORS

Solution.	Approximate normality.	Approximate H Ion concentration.	1 Tropaeolin 00 (‡)	2 Methyl Orange.	3 Töpfer's Reagent.	4 Congo Red.	5 Alizarin.	6 Litmus.	7 Neutral Red.	8 Phenolphthalein.	9 Günzberg's Reagent.	10 Boas' Reagent.	11 Tropaeolin 00 (evaporation test).
1. 0.4 per cent HCl	N/10	1×10^{-1}											
2. 0.04 per cent HCl	N/100	1×10^{-2}											
3. 0.5 per cent acetic acid	N/10	1×10^{-3}											
4. Acid phosphate 1 : 9*	M/15	1×10^{-6}											
5. Water	.....	1×10^{-7}											
6. Basic phosphate 20 : 1*	M/15	1×10^{-8}											
7. Borate—NaOH 6 : 4†	N/10	1×10^{-10}											
8. NaOH 0.4 per cent	N/10	1×10^{-13}											

* Make up a solution of potassium dihydrogen phosphate of 1/15 molecular strength (9.078 gms. to a liter of water) and one of disodium hydrogen phosphate (obtained by drying the ordinary salt in the air for a few weeks), $Na_2HPO_4 \cdot 2H_2O$ of similar strength (11.876 gms. to a liter). To prepare the acid phosphate solution used in the test mix 1 part of the solution of the disodium salt with 9 parts of the solution of the dihydrogen phosphate. For the basic phosphate solution the proportions are 20 : 1.

† Borate—NaOH solution. Prepare a borate solution by dissolving 12.404 gms. of pure boric acid (0.2 mol) in 100 c.c. N NaOH solution and dilute with water to a liter. Prepare the Borate-NaOH solution by mixing 6 parts of the borate solution with 4 parts of N/10 NaOH.

‡ Indicator solutions. *Tropaeolin 00*, 0.05 gm. in 100 c.c. 50 per cent alcohol. *Methyl orange*, 0.1 gm. in 100 c.c. water. *Töpfer's reagent*, 0.5 gm. dimethyl-amino-azo-benzene in 100 c.c. 95 per cent alcohol. *Congo red*, 0.5 gm. in 90 c.c. water and add 10 c.c. 95 per cent alcohol. *Alizarin*, 1 gm. sodium alizarin sulphonate in 100 c.c. water. *Litmus*, preferably azo-litmin 1 per cent solution in water. *Neutral red*, 0.05 gm. in 50 c.c. 95 per cent alcohol, add 50 c.c. water. *Phenolphthalein*, 1 gm. in 100 c.c. 95 per cent alcohol.

ACIDIMETRY AND ALKALIMETRY[1]

This covers the analysis of acids and bases. For the quantitative determination of an acid solution it is necessary to start with an alkali of known strength and *vice versa*. In acidimetry and alkalimetry use is made of what is known as *normal* solutions.

Normal Solutions

A normal solution is one which contains in a liter the quantity of active ingredient which will replace, combine with or oxidize 1 gm. of hydrogen. Or again a normal solution is one which contains one "gram-equivalent" of the active reagent dissolved in a liter of solution. By "gram-equivalent" is meant the amount of substance corresponding to one gram-atom (1.008 gms.) of hydrogen. For convenience in computation the concentration of solutions used for volumetric purposes are expressed in terms of their normality, i.e., a solution is 2 normal, $\frac{1}{2}$ normal, $\frac{1}{10}$ normal, etc. The letter N is used as an abbreviation for *normal*.

The essential point in the employment of normal solutions is that one normal solution is equivalent to any other normal solution. Thus 1 c.c. $\frac{1}{10}$ N HCl is equivalent to 1 c.c. $\frac{1}{10}$ N KOH is equivalent to 1 c.c. $\frac{1}{10}$ N sulphuric acid, because each is equivalent to an identical quantity of hydrogen. It follows, therefore, that if one has a normal solution of either an acid or an alkali unknown strengths of other acids and alkalies may be determined with ease.

Since the molecular weight of sodium hydroxide (NaOH) is 40 and that of hydrochloric acid (HCl) is 36.46, it follows that 40 gms. of the former contain the same number of molecules as 36.46 gms. of the latter. If 40 gms. of sodium hydroxide and 36.46 gms. of hydrochloric acid are each dissolved in pure water sufficient to make one liter of solution, each liter will contain the same number of dissolved molecules.

Mixing equal volumes of two such solutions is therefore the same as bringing together practically the same number of the two kinds of molecules, and the result is the instantaneous and essentially complete transformation into sodium chloride (and water).

$$X\ NaOH + X\ HCl = X\ NaCl + X\ H_2O.$$

[1] Adapted from Folin: A Laboratory Manual of Biological Chemistry, 1919.

If either or both of the solutions should first be diluted with a considerable bulk of pure water, the result on mixing the two would be the same, for the extra amount of water present takes no part in the reaction (except to the extent of absorbing a part of the heat set free).

The two solutions are equivalent. They also happen to be normal solutions. The hydrochloric acid is normal because it contains 1 gm. of active or replaceable hydrogen per liter of solution, and not because it contains the same number of grams of HCl per liter as there are units in the molecular weight. The sodium hydroxide solution is normal because it is equivalent to a solution containing 1 gm. of replaceable hydrogen per liter.

The molecular weight of sulphuric acid is 98. A sulphuric acid solution containing exactly 98 gms. per liter contains therefore the same number of molecules per unit volume as the sodium hydroxide solution containing 40 gms. per liter. But 1 molecule of sulphuric acid requires 2 molecules of sodium hydroxide for the formation of the neutral salt, sodium sulphate, because the sulphuric acid molecule has 2 replaceable hydrogen atoms. The solutions are not equivalent, for the sulphuric acid contains 2 gms. active hydrogen per liter. It is exactly twice as strong as the sodic hydrate solution; it is a 2 normal solution.

On the basis of the above description of what constitutes a normal solution, calculate the number of grams per liter in tenth normal sulphuric acid (.1 N H_2SO_4), fifth normal hydrochloric acid (.2 N HCl), half normal oxalic acid (.5 N $C_2H_2O_4$, $2H_2O$), fourth normal acetic acid (.25 N CH_3COOH), half normal sodic hydrate (.5 N NaOH), twentieth normal barium hydrate (.05 N $Ba(OH_2)$, fifth normal ammonium hydrate (.2 N NH_4OH).

The same description of normal solutions applies to other substances than acids and alkalies, as for example, oxidizing and reducing substances such as potassium permanganate, potassium bichromate, iodine, cupric hydrate, stannous chloride. A normal solution is here one capable of liberating 1 gm. of reducing hydrogen (or of giving off exactly sufficient oxygen to oxidize 1 gm. of hydrogen) per liter. Potassium permanganate, for example, in the presence of sulphuric acid and some easily oxidizable substance is decomposed as follows:

$$2KMnO_4+3H_2SO_4=K_2SO_4+2MnSO_4+3H_2O+5O.$$

As the 2 permanganate molecules liberate oxygen enough for 10 hydrogen atoms it takes only one-fiftieth of the molecular weight expressed in grams (3.156 gms.) to make 1 liter of tenth normal solution.

The calculation of what constitutes normal or equivalent solutions of any reagent is not very difficult provided the equation representing the chemical reaction involved is thoroughly clear.

All the common mineral acids and strong alkalies contain so much water that it is in practice not feasible to weigh out with sufficient accuracy the theoretical quantity required for a standard solution of acid or alkali. The carbonates of sodium or calcium (or the carbonates of sodium or potassium, obtained by ignition of the corresponding oxalates) give exceedingly accurate results. Oxalic acid is very serviceable as starting material for the preparation of standardized solutions of acids and alkalis if it is pure and has lost none of its water of crystallization.

1. **Preparation of .5 N Oxalic Acid** (500 c.c.).—The usefulness of oxalic acid as a starting point for the preparation of standard acids and alkalies is due entirely to the fact that it can be obtained chemically pure and in condition suitable for direct weighing. Oxalic acid is, however, not a strong enough acid to titrate well with all the common indicators, and it is therefore not serviceable for acidimetric titrations in general. But by means of oxalic acid and with phenolphthalein as indicator, standard solutions of a strong alkali, like caustic soda, can be obtained, and by means of the latter standard solutions of the stronger mineral acids can then be prepared.

The reason why the strong acids and alkalies give more accurate and reliable results is the fact that the salts which they form when neutralized are not appreciably hydrolyzed by water into acid and base, as are the corresponding salts of the weaker acids and bases. The zone of neutrality to different indicators is therefore more sharply defined, and corresponds more nearly to the point represented by the presence of exactly equivalent amounts of acid and alkali.

Weigh accurately (to the fourth decimal) a small, clean, and dry beaker or large crucible. Then add to the weights on the balance pan 15.7560 gms. and add oxalic acid to the vessel on the other side until exact equilibrium is reached. Dissolve in distilled water this oxalic acid without the loss of a single crystal.

The acid dissolves rather slowly. The solution is therefore best made in a beaker with the aid of gentle heating with about 250 c.c. water. Transfer every drop of the solution to a measuring flask (500 c.c.), carefully rinsing the last traces from the beaker into the flask by means of successive small amounts of cold distilled water. Cool the flask in running tap water until the contents of the flask have reached the room temperature. (If a thermometer is used it must be rinsed carefully before it is removed from the flask.) Fill up with water until the lower side of the " meniscus " is exactly even with the 500 c.c. mark. Stopper the flask, and invert several times (30 to 40) so that the solution is thoroughly mixed. Transfer to a clean, *dry* bottle; label and preserve.

Using a strong base like sodium hydroxide and a sensitive indicator like phenolphthalein for the titration, it is possible to obtain quite reliable and accurate results with oxalic acid. The volumetric determinations involved in metabolism studies and urine analyses are, however, extensively based on titrating ammonia, which is a very weak base. Phenolphthalein, because of its high degree of sensitiveness to weak acids and its lack of sensitiveness to weak bases, is useless in titration of ammonia. The oxalic acid and the phenolphthalein are therefore used only for the purpose of securing a standard alkali solution.

2. **Preparation of .5 N Sodic Hydrate (1000 c.c.).**—The sodic hydrate used for standard solutions must be as free as possible from carbonates, because otherwise the solutions will not have the same titrating value with all the common indicators. Sodic hydrate absorbs rapidly carbonic acid from the atmosphere. The carbonic acid should first be removed from the alkali, and the solutions must afterwards be protected from too much exposure to the carbonic acid of the air. As the carbonates are almost insoluble in very strong sodic hydrate solutions, 40 per cent solutions in which the carbonates have settled can be used as a starting point.

Transfer about 80 c.c. clear 40 per cent sodic hydrate solution to a large flask or bottle, and add 1000 to 1200 c.c. water. Dissolve about 2 gms. barium hydroxide in about 100 c.c. hot water, and without filtering pour this solution into the sodic hydrate solution. Cover with a watch-glass, and set aside over night. By means of a siphon or compressed air remove the clear, supernatant solution to another flask.

To determine the exact value of the solution it is only necessary to find out how much of it is required for the neutralization of a known volume of the .5 N oxalic acid solution. With a dry and clean pipette transfer 25 c.c. of the oxalic acid into a beaker or flask. Dilute it by adding 100 to 150 c.c. of water, and add two drops of the indicator (1 per cent alcoholic solution of phenolphthalein).

Fill a dry, clean burette with the alkali and cover with a test-tube. After adjusting the solution in the burette to the zero mark, run it into the diluted oxalic acid, more and more cautiously toward the end until finally one single drop produces a deep red coloration. Note the volume of alkali required (to within .05 c.c.). Repeat the titration until two successive ones give exactly the same figure.

In titrating acids and alkalies the alkali must always be run into the acid solution, not vice versa.

From the result obtained calculate how much of the alkali would be required to neutralize 1 liter of the oxalic acid solution. By means of a 100 c.c. pipette and a burette, transfer the required amount of the alkali to the liter measuring flask, and fill up to the mark with water. Mix, transfer to a dry bottle, label, and stopper with a rubber stopper.

As a check on the work determine the concentration of an unknown hydrochloric acid solution (furnished), using as indicator (*a*) phenolphthalein, (*b*) alizarin red (2 drops 1 per cent aqueous solution).

3. **Half Normal Hydrochloric Acid.**—Concentrated hydrochloric acid is approximately 10 N solution of HCl. From it prepare 1000 c.c., .5 N solution, using the half normal sodium hydroxide as a standard and alizarin red as indicator. The most convenient way is first to prepare 1200 to 1300 c.c. of a solution somewhat stronger than half normal, and then, on the basis of titrations with the standard alkali, to dilute the required amount with water to 1 liter. One liter of half normal hydrochloric acid is enough for all the analytical work described in this manual.

Those who have had experience in volumetric analysis may use simply standardized solutions instead of the half normal in the case of hydrochloric acid and in the case of all other standard solutions, but beginners should not omit the preparation of a strictly half normal acid.

4. **Tenth Normal Acid and Alkali.**—From the half normal stock solutions of hydrochloric acid and sodium hydroxide, prepare 1000 c.c., .1 N hydrochloric acid and .1 N sodium hydroxide. The solutions so obtained should be equivalent.

PREPARATION OF N/10 HYDROCHLORIC ACID

Hulett and Bonner [1]

This extremely accurate method depends on the fact that when hydrochloric acid solution is distilled at 760 mm. pressure the concentration of HCl in the undistilled portion approaches 20.24 per cent. When this is reached further distillation yields a distillate also containing HCl of this concentration. To prepare stock HCl solution for standards, add to concentrated HCl (sp. gr. 1.2) an equal volume of water and bring to a density at 25° of 1.096 (see " Pycnometers ") by addition of more water or concentrated HCl. Distill off three-quarters of this mixture. The remaining one-quarter has within 1 part in 10,000 the following composition:

Barometric pressure at Distillation.	Per cent HCl.	Grams of Solution to make 1 liter of N/10 HCl.
770	20.218	18.04
760	20.242	18.02
750	20.266	18.00
740	20.290	17.97
730	20.314	17.95

STANDARDIZATION OF OXIDIZING SOLUTIONS

General Principle.—Many substances exist in two states of oxidation; i.e., iron in ferrous sulphate ($FeSO_4$), and ferric sulphate $Fe_2(SO_4)_3$, or arsenic in sodium arsenite (Na_3AsO_3) and sodium arsenate (Na_3AsO_4), etc. These substances may readily

[1] Medical War Manual, No. 6, 1918, p. 78.

be changed from the lower to the higher state by the commonly employed oxidizing agents, $KMnO_4$, I_2 or $K_2Cr_2O_7$.

$$10FeSO_4+2KMnO_4+8H_2SO_4 = 5Fe(SO_4)_3+K_2SO_4+2MnSO_4+8H_2O.$$

$$Na_3AsO_3+I_2+H_2O = Na_3AsO_4+2HI.$$

In a similar manner, many organic compounds yield readily to decomposition.

$$5H_2C_2O_4+2KMnO_4+3H_2SO_4 = 10CO_2+8H_2O+K_2SO_4+2MnSO_4.$$

$$CO(NH_2)_2+3I_2+6NaOH = CO_2+N_2+6NaI+5H_2O.$$

It is apparent that if the strength of the oxidizing solution is known (that is, if the oxidizing solution is standardized) and the volume used up in the reaction noted, we can calculate from these equations the quantity of the substance oxidized or decomposed. In this way standardized oxidizing solutions find a wide use in the quantitative determination of oxidizable compounds.

Oxidizing Agents.—The common oxidizing agents employed are $KMnO_4$, I_2, and $K_2Cr_2O_7$. We shall consider only the first two. $KMnO_4$ gives up its oxygen directly.

$$2KMnO_4 = K_2O\cdot 2MnO\cdot 5O.$$

This reduction is always carried on in the presence of H_2SO_4 in order to dissolve the K_2O and the colored MnO.

$$K_2O+2MnO+5O+3H_2SO_4 = K_2SO_4+2MnSO_4+3H_2O+5O.$$

(Keep in mind that 2 molecules of $KMnO_4$ yield 5 atoms of Oxygen.)

Iodine yields oxygen indirectly.

$$I_2+H_2O = 2HI+O.$$

(Keep in mind that 2 atoms of Iodine yield 1 of Oxygen.)

The Normal Solution.—Although these reactions are complex, the strength of the reagents may nevertheless be expressed in simple

terms. The basis selected is oxygen and the strength of all standard solutions is expressed in terms of available oxygen. A normal oxidizing solution is defined as one yielding 8 gms. of available oxygen per liter. Since 8 gms. of oxygen is the equivalent of 1 gm. of H, this definition agrees with the general definition of a normal solution (a solution yielding 1 gm. H per liter).

(*a*) *Potassium Permanganate.*—As observed above 2 molecules of $KMnO_4$ yield 5 available atoms of Oxygen (equivalent of 10 H). By definition, therefore, a normal solution of $KMnO_4$ contains

$$\frac{2KMnO_4}{10} = \frac{2 \times 158.03}{10} = 31.606 \text{ gms. per liter.}$$

(*b*) *Iodine.*—As observed above I_2 yields 1 atom of Oxygen (equivalent of 2H). Hence by definition a normal solution of Iodine contains

$$\frac{2I}{2} = \frac{2 \times 126.92}{2} = 126.92 \text{ gms. per liter.}$$

Indicators.—It is of course necessary to know when the oxidation is complete or in other words when the oxidizing agent has been completely reduced. $KMnO_4$ serves as its own indicator because as it is reduced, its purple color disappears and the first drop added in excess turns the solution a permanent pink.

Iodine may also serve as its own indicator since its color ranges from deep red to yellow, but a far better end-point is obtained by employing starch as an indicator. As the last trace of iodine is used up the blue of the starch iodine reaction disappears and the solution becomes colorless.

Preparation of Solutions. (*a*) *Potassium Permanganate.*—Whereas it is possible to weigh out the $KMnO_4$ accurately and dissolve it in the proper amount of water, the practice is a poor one unless certain precautions are observed, for there are several factors that tend to weaken the solution shortly after it is made up. One is the presence of the impurity MnO_4 even in the best preparations on the market, which reduces the $KMnO_4$ at an increasing rate as the solution grows older. Others are the presence of NH_3 in the purest distilled water, grease in the glassware, etc. It

is better therefore to make up a stronger solution than the one planned and to let it stand for some two weeks until all the impurities have been oxidized, and then to standardize it against a stable reagent, oxalic acid for example. However, when in a hurry the solution can be standardized at once if the following is observed: The glassware should be cleaned in dichromate cleaning mixture. The water should be boiled with a few crystals of $KMnO_4$ in it, to oxidize the organic matter. After the solution is finally made up, it should be filtered through asbestos that has been previously treated with aqua regia to remove the organic matter. This filtration removes the insoluble MnO_4.

Weigh out 1.75 gms. $KMnO_4$ (for $\frac{1}{2}$ liter of N/10 solution) and dissolve in the specially prepared water. Then filter the solution through the specially prepared asbestos and dilute to the mark. This solution is ready for standardization against N/10 oxalic acid.

(*b*) *Iodine.*—It is impracticable to weight out iodine accurately because it sublimes so readily. It is therefore dissolved in strong KI solution, in excess of the theoretical amount, diluted to volume and then standardized against sodium thiosulphate solution.

Weigh out 7 gms. Iodine (for $\frac{1}{2}$ liter of N/10 solution). Dissolve 10 gms. KI in 5 c.c. of water (*no more*) and then dissolve the iodine in the concentrated KI solution. Use only glass stoppers for the bottles.

(*c*) *Oxalic Acid.*—$KMnO_4$ is standardized against oxalic acid as has been said. It will be necessary therefore to prepare an N/10 solution. This can readily be made by diluting the N/2 oxalic acid solution that you have already prepared.

(*d*) *Sodium Thiosulphate.*—An N/10 solution of $Na_2S_2O_3$ is used to standardize the Iodine solution according to the equation

$$2Na_2S_2O_3 + I_2 = Na_2S_4O_6 + 2NaI.$$

Since 2 molecules of the thiosulphate is equal to 2 atoms of iodine a normal solution of $Na_2S_2O_3$ is equal to

$$\frac{2Na_2S_2O_3 \cdot 5H_2O}{2} = \frac{2(248.20)}{2} = 248.20 \text{ gms. per liter.}$$

Weigh out 13 gms. $Na_2S_2O_3$ and dissolve in 500 c.c. CO_2 free water. The carbonic acid attacks the thiosulphate weakening the

solution. The water is therefore boiled and kept in bottles closed by a soda lime tube. It is drawn off by a syphon.

Standardization.—The standardizations are carried on in this order.

$KMnO_4$ against N/10 oxalic acid.

$Na_2S_2O_3$ against N/10 $KMnO_4$.

Iodine against N/10 $Na_2S_2O_3$.

(*a*) *Potassium Permanganate.*—Measure out 25 c.c. N/10 oxalic acid in a beaker and add 10 c.c. of diluted H_2SO_4 (1:4). Then dilute with 200 c.c. water at 70°. The heat aids the destruction of the oxalic acid. But a higher temperature may cause a volatilization of the oxalic acid. Run in the permanganate solution from a burette until the first drop in excess produces a permanent pink.

$$5H_2C_2O_4+2KMnO_4+3H_2SO_4 = K_2SO_4+2MnSO_4+10CO_2+8H_2O,$$

or

$$5H_2C_2O_4+5O=10CO_2+5H_2O.$$

From these equations it is apparent that 1 molecule of oxalic acid is equivalent to 1 atom of oxygen. A normal solution of oxalic acid is therefore equivalent to a normal solution of $KMnO_4$, hence the calculation is c.c. for c.c. From the calculated strength figure out the amount of dilution necessary to make an exactly N/10 $KMnO_4$ solution.

(*b*) *Sodium Thiosulphate Solution.*—Place 1 gm. of KI in a beaker and dissolve it in 8 c.c. of water. To this add 5 c.c. HCl solution (1:5) and 25 c.c. of N/10 $KMnO_4$. Dilute to 200 c.c. The $KMnO_4$ oxidizes the HCl to release chlorine which replaces the Iodine in the KI.

$$2KMnO_4+16HCl=2KCl+2MnCl_2+3H_2O+10Cl.$$

$$10Cl+10KI=10KCl+10I.$$

Run in the thiosulphate solution from a burette until the iodine color becomes pale yellow. Then add 2 c.c. of starch solution. The first drop that causes the blue color to disappear

marks the end-point. The calculation is again c.c. for c.c. From the calculated strength figure out the amount of dilution necessary to make an exactly N/10 $Na_2S_2O_3$ solution.

(*c*) *Iodine Solution.*—Measure out 25 c.c. of iodine solution in a beaker and dilute with 200 c.c. water. Run in the N/10 thiosulphate solution from a burette until the iodine color becomes pale yellow. Then add 2 c.c. starch solution; the first drop that causes the blue color to disappear marks the end-point. The calculation is again c.c. for c.c. From the calculated strength figure out the amount of dilution necessary to make an exactly N/10 iodine solution.

PART II

METHODS FOR THE ANALYSIS OF URINE

ACIDITY OF URINE BY TITRATION

Folin's Method [1]

Principle.—The urine is titrated with standard sodium hydroxide solution, using phenolphthalein as an indicator. Potassium oxalate is added to precipitate the calcium which would otherwise interfere with the end-point due to the precipitation of calcium phosphate on neutralization of the urine. The acidity of the urine as determined in this way is not a correct measure of the true acidity, which is dependent upon the concentration of hydrogen ions. The results obtained do, however, ordinarily show a certain parallelism with the hydrogen ion concentration and are of value for comparative purposes.

Procedure.—Place 25 c.c. of urine in a 200 c.c. Erlenmeyer flask or beaker and add 15–20 gms. of finely pulverized neutral potassium oxalate and 1 to 2 drops of a 1 per cent phenolphthalein solution to the fluid. Shake the mixture vigorously for 1 to 2 minutes and titrate it immediately with N/10 sodium hydroxide until a faint but unmistakable pink remains permanent on further shaking. Take the burette reading and calculate the acidity of the urine under examination.

Calculation.—If y represents the number of cubic centimeters of N/10 sodium hydroxide and used y' represents the volume of urine excreted in twenty-four hours, the total acidity of the

[1] Folin: Am. Jour. Physiol., 1905, 13, 45.

twenty-four hour urine specimen may be calculated by means of the following proportion:

$25 : y :: y' : x$ (acidity of twenty-four hour urine expressed in cubic centimeters of N/10 sodium hydroxide).

Each cubic centimeter of N/10 sodium hydroxide contains 0.004 gm. of sodium hydroxide, and this is equivalent to 0.0063 gram of oxalic acid. Therefore, in order to express the total acidity of the twenty-four hour urine specimen in equivalent grams of sodium hydroxide, multiply the value of x, as just determined, by 0.004, or multiply the value of x by 0.0063 if it is desired to express the total acidity in grams of oxalic acid.

THE TITRATION OF ORGANIC ACIDS IN URINE

Method of Van Slyke and Palmer[1]

Principle.—Interfering substances (carbonates and phosphates) are removed from the urine and the remaining weak acids titrated. Creatinine is included and a correction for its presence must be made.

Procedure.—100 c.c. of urine, roughly measured, are thoroughly mixed with 2 gms. of finely powdered calcium hydroxide, allowed to stand about fifteen minutes with occasional stirring, and then passed through a dry folded filter. This treatment removes carbonates and phosphates. To 25 c.c. of the filtrate in a 125 to 150 c.c. test-tube of clear glass (Pyrex, 30 mm. $\times$200 mm.) one adds 0.5 c.c. of 1 per cent phenolphthalein solution, and 0.2 N hydrochloric acid from a burette (amount need not be measured) until the pink color just disappears (pH = approximately 8). 5 c.c. of 0.02 per cent tropaeolin 00 solution are then added. As the indicator solution is added it is thoroughly mixed with the urine by shaking the tube; if this precaution is omitted some of the tropaeolin 00 may be precipitated. Finally 0.2 N hydrochloric acid is added from the burette until the red color equals that of a standard solution containing 0.6 c.c. of 0.2 N HCl, 5 c.c. of tropaeolin 00 solution, and water to a total volume of 60 c.c. When

[1] Van Slyke and Palmer: Jour. Biol. Chem., 1920, 41, 567.

the end-point is approached, sufficient water is added to the titrated solution to make its volume equal to that of the 60 c.c. standard solution used in a similar tube as a color control.

In comparing the color of the titrated solution with that in the standard, it is convenient during the titration to hold the two tubes side by side between the thumb and fingers, the tube containing the urine being the one held nearer to the tips of the fingers where it can be easily shaken as the 0.2 N acid is run in from the burette.

Sometimes it is desirable to use a similar technique for the phenolphthalein end-point also. In this case a tube of urine filtrate to which no phenolphthalein is added serves as a standard.

Calculation.—From the volume of 0.2 N HCl used to titrate from the end-point of phenolphthalein to that of the tropaeolin 00, the amount, usually 0.7 c.c. is subtracted which is utilized in a similar titration of a control determination in which water is substituted for the urine. The volume of 0.2 N HCl thus corrected represents the approximate organic acid content of the urine sample, plus the creatine and creatinine, and an amount of amino-acids ordinarily negligible.

In order to calculate the results in terms of c.c. of 0.1 N organic acid per liter, the figure representing the c.c. of 0.2 N HCl used in the titration is multiplied by 80 (by $\frac{1000}{25}=40$ in order to transfer figure from 25 c.c. to 1000 c.c. of urine, and by 2 to change from 0.2 N to 0.1 N terms).

Correction for Creatinine.—A 0.1 N solution of creatinine (11.32 mg. per c.c.) titrates in the above determination as a 0.1 N solution of organic acid. Therefore, in order to correct for the creatinine, the c.c. of 0.1 N organic acid per liter calculated from the above titration may be diminished by

$$\frac{\text{mg. creatinine per liter urine}}{11.32} \text{ or by } \frac{\text{mg. creatinine N per liter urine}}{4.2}.$$

The simplest way is to subtract the creatinine correction directly from the c.c. of 0.2 N acid used in the titration, and multiply the difference by 80. In this case the correction is $\frac{1}{80}$ as great as the above; i.e., c.c. correction =

$$\frac{\text{mg. creatinine per liter urine}}{906} \text{ or } \frac{\text{mg. creatinine N per liter urine}}{336}.$$

Example.

0.2 N HCl used in titration		7.6 c.c.
Correction found in blank analysis	0.7 c.c.	
Creatinine correction for 500 mg. creatinine N per liter urine. Correction = 500 c.c. =	1.2 c.c.	
Total correction	1.9 c.c.	
Corrected titration figure = 7.6 − 1.9		5.7
0.1 N organic acid per liter = 80 × 5.7		456.0

Remarks.—Tropaeolin 00 is preferred as indicator for the final end-point. In neutral solution it gives nearly the same yellow color as urine, but so much more intense that a water solution of 0.002 N hydrochloric acid with the indicator can be used as a color standard. Very dark urines may need greater dilution. Another advantage of this indicator is that its maximum acid color is not reached even at pH 2.7, so that if too much HCl is added in the titration the solution becomes redder than the standard. This particular advantage is possessed in much less degree by the three indicators mentioned below as alternatives.

Other indicators that may be used are methyl orange, tetrabromophenolsulfonephthalein (bromophenol blue, Clark and Lubs), and dimethylaminoazobenzene. To some eyes the color change of one of these dyes may be more readily detected than that of tropaeolin 00. The two azo dyes are not much different in color from tropaeolin 00, both changing from yellow to red, but the bromophenol blue turns from blue to a clear yellow on acidifying, and affords a very different alternative. The tropaeolin 00 end-point appears, however, the most satisfactory.

Theoretical Basis of Method.—The method is based on the following previously known facts:

1. Relatively little strong mineral acid is required to change the hydrogen ion concentration of a water solution from 10^{-3} to 2×10^{-3} if the only electrolytes present are alkali salts of strong acids, such as sulfates and chlorides.

2. If the salt of a weak acid is present, however, the addition of nearly a full molecule of hydrochloric acid for each molecule of such salt is necessary in order to cause the above change in hydrogen ion concentration. The organic acids known to occur in normal and pathological urines, in amounts sufficient to be quantitatively significant in the total acid excretion of the body, belong to the class of weak acids whose salts behave in the above manner.

3. The only mineral acids found in significant amounts in urine which belong to the class of weak acids, and therefore form salts which show the above behavior, are phosphoric and carbonic acids.

4. Very weak bases form salts which behave like those of the weak acids. Creatinine is titrated almost quantitatively in changing the hydrogen ion concentration from 10^{-3} to 2×10^{-3}, and creatine to about 60 per cent. Aside from the traces of amino-acids, these appear to be the only bases of this kind present in considerable amount in human urine.

HYDROGEN ION CONCENTRATION OR TRUE ACIDITY

Indicator Method

(Henderson and Palmer's Adaptation of Sorensen's Method)[1]

Principle.—The reaction of the urine is estimated by matching the colors produced when a few drops of indicator are added respectively to the diluted urine and to standard solutions of known reaction similarly diluted. Similar hydrogen ion concentrations are indicated by similar colors. The indicator must be properly chosen.

Standard Solutions.—A series of standard solutions of known hydrogen ion concentration must be prepared. The solutions as indicated in Table I (page 26) are satisfactory for urine analysis. The table also indicates the H ion concentration of each solution, the figure given being the logarithm of this concentration (P_{H+}). It is more convenient and rational to express the concentration by this logarithmic notation. True H ion concentrations corresponding to the logarithmic figures are given in Table II (page 26).

The thirteen solutions indicated are made up by mixing equal volumes of their ingredient solutions of the composition indicated. Solutions 4 to 12 are all that are ordinarily required, as the normal urinary H ion concentrations lie between 4.80 and 7.50 and pathological variations are usually within these limits. The mean normal value is almost exactly 6.00.

[1] Henderson and Palmer: Jour. Biol. Chem., 1913, 13, 393.

TABLE I

No.	NaH_2PO_4	Na_2HPO_4	P_{H+}	Indicator
1		0.1000 N	9.27	
2	0.0001 N	0.0480 N	8.7	Phenolphthalein
3	0.0001 N	0.0120 N	8.0	
4	0.0166 N	0.0833 N	7.48	Neutral red
5	0.0010 N	0.0060 N	7.38	
6	0.0010 N	0.0023 N	6.90	
	CH_3COOH	CH_3COONa		
7	0.0009 N	0.0920 N	6.70	
8	0.0023 N	0.0920 N	6.30	Sodium alizarine sulphonate
9	0.0046 N	0.0920 N	6.00	
10	0.0092 N	0.0920 N	5.70	*p*-Nitrophenol
11	0.0230 N	0.0920 N	5.30	
12	0.0460 N	0.0920 N	4.90	Methyl red
13	0.0920 N	0.0920 N	4.70	

TABLE II

Log.	$\frac{+}{H}$	Log.	$\frac{+}{H}$
4.6	250×10^{-7}	6.4	4.0×10^{-7}
4.8	160×10^{-7}	6.6	2.5×10^{-7}
5.0	100×10^{-7}	6.8	1.6×10^{-7}
5.2	63×10^{-7}	7.0	1.0×10^{-7}
5.4	40×10^{-7}	7.2	0.63×10^{-7}
5.6	25×10^{-7}	7.4	0.40×10^{-7}
5.8	16×10^{-7}	7.6	0.25×10^{-7}
6.0	10×10^{-7}	7.8	0.16×10^{-7}
6.2	6.3×10^{-7}	8.0	0.10×10^{-7}

Procedure.—Select thirteen large test-tubes of good glass and indistinguishable in color and form. Into each of ten of these introduce 10 c.c. of the various standard solutions. Make up to 60 c.c. with distilled water and add to each exactly the same amount of an aqueous solution of sodium alizarine sulphonate (10 to 15 drops). Mix well by inverting. Introduce 10 c.c. of the urine to be tested into a similar test-tube, dilute and add indicator in exactly the same way as before. Match the color of the diluted

urine solution with one of the standard solutions. By consulting Table II determine to what H ion concentration this corresponds. This table points out the indicators to be used for different ranges of acidity. From 5.3 to 6.7 *p*-nitrophenol is satisfactory and is used in the same way as alizarine except that it must be present in concentration of 0.08 per cent. Neutral red is used in the same way for acidities from 6.7 to 7.5 about 1.5 c.c. of the 1 per cent solution being required. For acidities greater than 5.5 methyl red is used in the following way: 10 c.c. portions of the standard solutions are introduced into carefully selected colorless test-tubes and 10 c.c. of urine is introduced into another tube. The standard solutions are then colored to match the urine by the addition of small amounts of *p*-nitrophenol, methyl orange, alizarine or Bismarck brown. Then to standard solutions and urine add 0.15 c.c. of a saturated solution in 50 per cent alcohol, of methyl red and match the colors. For concentrations of 7.5 to 9.27 or less undiluted urine is matched in test-tubes against undiluted standard solutions, using phenolphthalein as an indicator (without previous coloration of standard solution). In all cases estimations are made in duplicate

HASKINS' MODIFICATION OF HENDERSON AND PALMER'S METHOD [1]

Principle.—The principle involved is the same as that of Henderson and Palmer except that permanent standards are substituted for the solutions in the latter.

Permanent Standards.—The artificial standards advocated by Haskins are made by adding amaranth and paranitrophenol in various proportions in a buffer mixture which contains KH_2PO_4 and K_2HPO_4, each being in one-tenth molar concentration. The buffer solution (P_H 6.8) prevents the trace of alkali which gradually dissolves out of the glass of the bottles from changing the color of the standards. This solution gives a decided yellow color when paranitrophenol is added, the intensity of the color depending upon the amount of paranitrophenol used. Amaranth imparts a brilliant red color, and is not affected by the phosphate.

The amaranth and paranitrophenol solutions should be kept

[1] Haskins: Jour. Lab. and Clin. Med., 1919, 4, 363.

in pyrex or nonsol flasks. They do not fade when exposed to diffused light of moderate intensity. The permanent standards do not change appreciably if properly cared for. They should not be kept standing in direct sunlight, and should be kept in a box or cupboard when not in use. Even after nine months the standards are in excellent condition, the color changes being so slight as to cause practically no error in estimation of the P_H of a urine.

Each standard is prepared to match somewhat closely the color given by 1 c.c. of a Sorensen's phosphate mixture diluted with 20 c.c. of boiled distilled water after the addition of 1 c.c. of indicator, when at ordinary room temperature (15° to 22°). Absolute matching of the colors is not at all necessary. The use of rosolic acid with urine is possible only with the aid of the permanent standards. Sorensen's phosphate standards with rosolic acid change color too rapidly for practical use. For these permanent standards 20 c.c. of the buffer phosphate solution is put into each of the two-ounce square bottles and various amounts of amaranth and paranitrophenol are added as indicated below, and a little preservative (0.2 c.c. 10 per cent thymol in chloroform) is added, then the bottle is corked (but not with a rubber stopper) and sealed with hot paraffin.

The exact P_H figures for the Sörensen phosphate mixtures are those given in the parentheses. The approximate figures can be used for most work, since it is impossible to detect differences between solutions that vary less than 0.05 in their P_H figures.

The concentrations of amaranth in indicator solutions are as follows: Amaranth (amaranth No. 107, Eimer & Amend), 8 mg. dissolved in 100 c.c. of distilled water to which is added 0.5 c.c. of the thymol-chloroform preservative.

Paranitrophenol (Eimer & Amend), 20 mg. dissolved in 10 c.c. of alcohol and diluted with 90 c.c. of distilled water.

Methyl red (Hynson, Wescott & Dunning), very finely powdered, 2.4 mg. dissolved in 100 c.c. of 60 per cent alcohol.

Rosolic acid (Hynson, Wescott & Dunning), powdered, 16 mg. dissolved in 100 c.c. of 95 per cent alcohol.

Neutral red (Hynson, Wescott & Dunning), finely powdered, 3.2 mg. dissolved in 100 c.c. of 50 per cent alcohol.

For these solutions the concentrations of alcohol need not be exact. The last three indicator solutions must be protected from the light, preferably by painting the flasks black. These indicator

solutions can still be used satisfactorily when two months old, although the rosolic acid weakens slightly in that time. New solutions should be prepared once in two or three months.

PERMANENT STANDARDS AND THE CORRESPONDING SÖRENSEN STANDARDS

Permanent Standards			Sörensen Standards		
			Proportions in 10 c.c. of mixture		
P_H	Amaranth, c.c.	Paranitrophenol, c.c.	1/15 Molar KH_2PO_4 c.c.	1/15 Molar Na_2HPO_4 c.c.	Indicator
4.5 (4.49)	1.3	0.2	10.0	0.0	Methyl red
4.95 (4.94)	1.0	0.45	9.9	0.1	Meythl red
5.3 (5.29)	0.65	0.75	9.75	0.25	Methyl red
5.6 (5.59)	0.45	1.1	9.5	0.5	Methyl red
5.9 (5.91)	0.25	1.0	9.0	1.0	Methyl red
5.9 (5.91)	0.55	1.0	9.0	1.0	Rosolic acid
6.25 (6.24)	0.7	0.9	8.0	2.0	Rosolic acid
6.45 (6.47)	0.9	0.85	7.0	3.0	Rosolic acid
6.65 (6.64)	1.0	0.65	6.0	4.0	Rosolic acid
6.8 (6.81)	1.15	0.3	5.0	5.0	Rosolic acid
7.0 (6.98)	1.3	0.4	4.0	6.0	Rosolic acid
7.0 (6.98)	0.6	0.45	4.0	6.0	Neutral red
7.15 (7.17)	0.6	0.65	3.0	7.0	Neutral red
7.4 (7.38)	0.43	0.6	2.0	8.0	Neutral red
7.5 (7.48)	0.38	0.65	1.65	8.35	Neutral red

Technic in Estimating P_H of Urine

All glassware used (bottles, pipettes, flasks, etc.) must be thoroughly washed, rinsed with distilled water, and then tested with a practically neutral indicator solution (e.g., 20 c.c. distilled water, 1 drop of the buffer phosphate solution and 1 c.c. of rosolic acid). If the test solution becomes less red, contaminating acid is present, if it becomes redder, alkali is present; in either case

wash the apparatus and test again. Rinse out the traces of indicator solution with distilled water and proceed with the estimation.

First make the preliminary determination as follows: To 20 c.c. of distilled water in a 2-ounce square bottle add 1 c.c. of rosolic acid solution and immediately add the urine 0.2 c.c. at a time comparing with the rosolic acid permanent standards after each addition until either the P_H determination remains constant or the urinary pigment changes the character of the color. Never use more than 1 c.c. of urine. In the case of highly concentrated urine 0.2 or 0.4 c.c. will suffice. This test indicates the amount to use in the final test, i.e., less than will give the disturbing pigment effect but still enough to give the full P_H estimation.

The test also shows which indicator must be used. If the mixture is as yellow as the 5.9 rosolic standard or more yellowish, use methyl red. If it is as red as the 7 rosolic standard or of a deeper red color, use neutral red. If the color is intermediate between that of the 5.9 and 7 standards, use rosolic acid.

For the final test add to 20 c.c. of distilled water the amount of urine determined by the first test, and last of all 1 c.c. of the proper indicator. Compare with the standards *immediately* (because contamination with carbon dioxide occurs so easily, and in the case of rosolic acid the color fades rapidly). Avoid getting expired air into the bottle.

It is self-evident that one must be careful to compare the urine mixture only with those standards that correspond to the particular indicator used (for instance, after using rosolic acid for the urine do not match up with the colors given by methyl red or neutral red). It is advisable to have each standard bottle labeled with the indicator, for example, Methyl R. 5.3, Ros. 6.8, Neutral R. 7.4; thus accidental slips will be avoided.

The P_H figure of the permanent standard that most nearly matches in color the urine test solution is taken as the P_H of the urine. One may guess P_H readings intermediate between the standards although such interpolation is an unnecessary refinement. The most accurate and most rapid estimations are made by placing the standard bottle and the urine bottle for comparison against a background of dull white paper with the light (diffused daylight or electric light) passing over the shoulder.

Remarks. 1. Toluene is to be added to the urine as a preservative when collecting a 24-hour sample, or when the urine cannot

be examined within an hour after being passed. The urine must not be treated in any other way, not even filtered. If it is turbid it may be centrifuged.

2. Urines containing hemoglobin, bile or great excess of pigment may be dialyzed in a collodion sac against neutral 1 per cent sodium chloride solution for thirty minutes, as in Rowntree's method for P_H of blood. The P_H of the salt solution is then determined in the same way as urine.

3. The room must be free from fumes of ammonia, hydrochloric or nitric acid when estimations are made. Excess of CO_2 (from flames) should be avoided.

4. For measuring amaranth and paranitrophenol solutions and also the urine, 1 c.c. pipettes marked in 0.01 c.c. are needed.

5. When the temperature of the room is above 22° an error may be made in estimation due to the temperature (the permanent standards do not change perceptibly), for instance, if the urine test mixture is at 30° the P_H figure will be greater (more alkaline) than at 20° by about 0.05 (in the case of a distinctly acid urine) up to even 0.20 (in the case of an alkaline urine). This can be obviated readily by cooling the distilled water to about 15° before using it.

6. The presence of protein in the urine does not interfere with the use of the indicator.

7. The phosphates for the preparation of the buffer solution must be pure for this purpose. Baker's C. P. KH_2PO_4 and Merck's pure K_2HPO_4 may be used. The buffer solution must have a P_H of 6.8 when estimated by the same dilution method as in the case of urine.

Dissolve each phosphate separately in about 200 c.c. of distilled water, taking 6.808 gms. of powdered KH_2PO_4 and 8.712 gms. of powdered K_2HPO_4. Mix the solutions and dilute to exactly 500 c.c., saturate with chloroform and filter. Keep the solution in a pyrex flask tightly corked.

8. A slight precipitate may appear after a time in the permanent standards, but this need not interfere with their use if they are not shaken up.

9. Test the indicator solution at least once a month by making P_H estimations with Sörensen's phosphate mixture (1 c.c. diluted as in estimating urine), using for methyl red the P_H 5.3 mixture and for neutral red the P_H 7.15 mixture. For rosolic acid the buffer phosphate mixture can be used (P_H 6.8). This procedure

also assures one that the permanent standards have not changed enough to cause inaccurate P_H estimations.

10. The distilled water may be tested at any time by adding to 20 c.c. two drops of the buffer phosphate solution and 1 c.c. of rosolic acid, the color must be redder than the 6.65 standard but not redder than the 6.8 standard. The distilled water should not be too old.

11. With these standards it is easy to make the estimations by artificial light, preferably using a daylight lamp.

12. For research accuracy it is necessary to use boiled distilled water. For ordinary routine work unboiled distilled water can be used, but it should be kept in a pyrex flask sealed with a rubber stopper *constantly* except when water is being poured out. Do not allow expired air to get into the flask. Excess of carbon dioxide in the water can change the estimation noticeably in the case of urines that are alkaline or very slightly acid.

TOTAL SOLIDS

1. **Drying Method.**—Place 5 c.c. of urine in a weighed shallow dish, acidify *very slightly* with acetic acid (1 to 3 drops), and dry it *in vacuo* in the presence of sulphuric acid to constant weight. Calculate the *percentage* of solids in the urine sample and the *total solids* for the twenty-four-hour period.

2. **Long's Coefficient.**—The quantity of solid material contained in the urine excreted for any twenty-four hour period may be approximately computed by multiplying the second and third decimal figures of the specific gravity by 2.6. This gives us the *number of grams of solid matter in* 1 *liter of urine.* From this value the total solids for the twenty-four-hour period may easily be determined.

Calculation.—If the volume of urine for the twenty-four hours was 1120 c.c. and the specific gravity 1.018, the calculation would be as follows:

(*a*) $18 \times 2.6 = 46.8$ gms. of solid matter in 1 liter of urine.

(*b*) $\frac{46.8 \times 1120}{1000} = 52.4$ gms. of solid matter in 1120 c.c. of urine.

Long's coefficient was determined for urine whose specific gravity was taken at 25° C. and is probably more accurate, for conditions obtaining in America, than the older coefficient of Haeser, 2.33.

TOTAL NITROGEN

Kjeldahl Method

Principle.—The principle of this method is the conversion of the various nitrogenous bodies of the urine into ammonium sulphate by boiling with concentrated sulphuric acid, the subsequent decomposition of the ammonium sulphate by means of a fixed alkali (NaOH) and the collection of the liberated ammonia in an acid of known strength. Finally, this partly neutralized acid solution is titrated with an alkali of known strength and the nitrogen content of the urine under examination computed.

Procedure.—Place 5 c.c. of urine in a 700 c.c. Kjeldahl flask, add 20 c.c. of concentrated sulphuric acid and about 0.2 gm. of copper sulphate, or 10 gms. of potassium sulphate and boil the mixture for some time after it is colorless (about one-half hour).

Allow the flask to cool and dilute the contents with about 200 c.c. of ammonia-free water. Add a little more of a concentrated solution of NaOH than is necessary to neutralize the sulphuric acid and introduce into the flask a little coarse pumice stone or a few pieces of granulated zinc, to prevent bumping, and a small piece of paraffin to lessen the tendency to froth. By means of a safety-tube connect the flask with a condenser so arranged that the delivery-tube passes into a vessel containing a known volume (the volume used depending upon the nitrogen content of the urine) of N/10 sulphuric acid, using care that the end of the delivery-tube reaches beneath the surface of the fluid. Mix the contents of the distillation flask very thoroughly by shaking and distill the mixture until its volume has diminished about one-half. Titrate the partly neutralized N/10 sulphuric acid solution by means of N/10 sodium hydroxide, or ammonium hydroxide, using Congo red as indicator, and calculate the content of nitrogen of the urine examined.

Calculation.—Subtract the number of cubic centimeters of N/10 sodium hydroxide used in the titration from the number

of cubic centimeters of N/10 sulphuric acid taken. The remainder is equivalent to the number of cubic centimeters of N/10 sulphuric acid, neutralized by the ammonia of the urine. One c.c. of N/10 sulphuric acid is equivalent to 0.0014 gm. of nitrogen. Therefore, if y represents the volume of urine used in the determination and y' the number of cubic centimeters of N/10 sulphuric acid neutralized by the ammonia of the urine, we have the following proportion:

$y : 100 : y' \times 0.0014 : x$ (percentage of nitrogen in the urine examined).

Calculate the quantity of nitrogen in the twenty-four-hour urine specimen.

A SIMPLIFIED KJELDAHL METHOD FOR URINE [1]

Principle.—The principle is identical with that for the ordinary Kjeldahl determination.

Procedure.—Transfer 5 c.c. of undiluted urine to a 300 c.c. Kjeldahl flask (Pyrex). Add 5 c.c. of the phosphoric-sulphuric acid mixture, also 2 c.c. of 10 per cent ferric chloride solution and four to six small pebbles to prevent bumping. Boil in a hood over a micro-burner. Boil vigorously. In three to four minutes the foam which forms at first will entirely disappear and the flask becomes filled with dense white fumes. When this stage is reached (but no earlier) cover the mouth of the flask with a small watch glass and continue the vigorous heating for two minutes. At the end of two minutes dilute urines will already be green or blue, and concentrated urines will be a light straw yellow, the black carbonaceous matter will be completely destroyed. The flame should then be turned very low and the gently boiling process should be continued for two minutes, making a total boiling period of four minutes, counting from the time the watch glass was put in place. Remove the flame and let the flask cool for four to five minutes. At the end of four, or not more than five minutes, add *first* 50 c.c. of water, then 15 c.c. of saturated sodium hydroxide (50 to 55 per cent), and connect the flask promptly, by means of a rubber stopper and ordinary

[1] Folin: Jour. Biol. Chem., 1919, 38, 461.

glass tubing, with a receiver containing from 35 to 75 c.c. of N/10 acid, together with water enough to make a total volume of 150 c.c., and a drop or two of alizarin red. Florence flasks, capacity 300 c.c. of Pyrex glass, make excellent receivers for this distillation. As soon as the connection is made with the receiver apply the flame again at full force but not directly under the center until the acid and alkali in the flask have had time to mix. The contents in the flask begin to boil almost at once and four to five minutes transfers the whole of the ammonia to the receiver. The contents in the receiver become heated, of course, since no condenser is used, but under the conditions described the temperature reached is only 65° to 70° C.

The only precaution needed in connection with the titration of the distillate (without previous cooling) is that a faint red color shall be accepted as the end-point. The color will deepen on cooling, and, if time permits, it is more satisfactory to cool in running water before titrating.

Remarks.—While the operator is directed to make prompt connections with the receiver after the alkali has been added to the digestion mixture, there is in point of fact very little danger of losing ammonia vapors by being unduly slow in closing the mouth of the flask. Similarly when the receiver contains too little acid and turns pink, there is no need of extraordinary haste in adding more acid. The water, though warm, will hold considerable free ammonia. The delivery tubes are made from glass tubing, small enough to pass into the ready-made holes in rubber stoppers. For the sake of flexibility the delivery tube should consist of two parts connected with a short piece of rubber tubing. This method is not applicable to highly resistant materials, as for example, milk; nor for urines containing much sugar. On the other hand fuming sulphuric acid may be substituted for ordinary sulphuric acid in the preparation of the hydrolyzing reagent, or if 2 c.c. of fuming sulphuric acid are used in addition to 5 c.c. of the regular reagent, sugar urines are readily destroyed within the regular heating period, from four to five minutes.

Preparation of the Hydrolyzing Reagent.—To 50 c.c. of 5 to 6 per cent copper sulphate solution add 300 c.c. of 85 per cent phosphoric acid and 100 c.c. of concentrated sulphuric acid. Five c.c. of this mixture are used for the destructive digestion of 5 c.c. of undiluted urine. Ten per cent solution of ferric chloride

is also required. The ferric chloride can scarcely be considered indispensable; but it hastens the digestion, and the iron hydroxide promotes even boiling during the subsequent distillation.

TOTAL NITROGEN

Folin-Farmer Microchemical Method [1]

Principle.—This method belongs with the so-called microchemical methods inasmuch as it is adapted to the determination of amounts of nitrogen in the neighborhood of 1 mg. while in the ordinary Kjeldahl procedure 30 to 100 mg. of nitrogen are generally manipulated. One c.c. of diluted urine is decomposed with sulphuric acid as in the Kjeldahl method, the ammonia formed is set free by the addition of alkali and carried over into an acid solution by means of a current of air. The ammonia solution is then treated with the Nessler-Winkler reagent and the color produced compared with that of a standard solution of an ammonium salt treated in the same way.

Procedure.—Introduce 5 c.c. of urine into a 50 c.c. volumetric flask if the specific gravity of the urine is over 1018, or into a 25 c.c. flask if the specific gravity is less than 1018. Fill the flask to the mark with distilled water and invert it several times in order to guarantee thorough mixing. Transfer 1 c.c. of the diluted urine to a large (20 to 25 mm. $\times$200 mm.) Jena-glass test-tube. Add to this 1 c.c. of concentrated sulphuric acid, 1 gm. of potassium sulphate, 1 drop of 5 per cent copper sulphate solution and a small, clean, quartz pebble or glass bead. (The pebble or bead is added to prevent bumping.) Boil the mixture over a micro-burner for about six minutes, i.e., about two minutes after the mixture has become colorless. Allow to cool until the digestion mixture begins to become viscous. This ordinarily takes about three minutes, but in any event the mixture must not be permitted to solidify. Add about 6 c.c. of water (a few drops at a time, at first, then more rapidly) to prevent solidification. To this acid solution add an excess of sodium hydroxide (3 c.c. of a saturated solution is sufficient) and aspirate the liberated ammonia by means of a rapid air current into a volumetric flask (100 c.c.) containing about 20 c.c. of ammonia-free water and 2 c.c.

[1] Folin and Farmer: Jour. Biol. Chem., 1912, 11, 493.

of N/10 hydrochloric acid. The air current should be only moderately rapid for the first two minutes but at the end of this two-minute period the current should be run at its maximum speed for an interval of eight minutes.

Disconnect the flask, dilute the contents to about 60 c.c. with ammonia-free water and dilute similarly 1 mg. of nitrogen in the form of ammonium sulphate in a second volumetric flask. Nesslerize both solutions as nearly as possible at the same time with 5 c.c. of Nessler-Winkler solution diluted, immediately before using, with about 25 c.c. of ammonia-free water to avoid turbidity. Immediately fill the two flasks to the mark with ammonia-free water, mix well and determine the relative intensity of the two colors by means of a Duboscq colorimeter.

The color of the unknown should be adjusted to that of the standard both from above and below the level of the latter. The matching of the colors is ordinarily very easy. It is desirable to make the readings by diffused daylight if possible. If electric light must be used, a sheet of smooth white paper should be interposed between the colorimeter and the source of light.

Calculation.—The reading of the standard divided by the unknown gives the nitrogen in milligrams in the volume of the urine taken. Calculate the total nitrogen output for the twenty-four-hour period.

THE COLORIMETER [1]

For this method as well as for a number of other methods commonly used in urinary and blood analysis an instrument known as a colorimeter is required. Through its aid we are able accurately to measure the respective depths of color in two solutions and hence to calculate the comparative amounts of substances which form colored compounds in a quantitative manner. The most satisfactory instrument for this purpose is the Duboscq colorimeter. This enables the two colored solutions to be compared in the same optical field and with a degree of accuracy of about 1 per cent. The later type of the Duboscq colorimeter with cylinders instead of prisms movable is to be preferred, particularly as this type may be readily adapted to the comparison of

[1] Hawk: Practical Physiological Chemistry, Sixth Edition, 1918.

cloudy solutions or suspensions, the instrument thus modified being called a nephelometer. In this later form of colorimeter the depths of the colored solutions through which the light passes are regulated by raising or lowering the cups and are accurately indicated in millimeters on a vernier scale at the back of the instrument. The standard solution is placed at any convenient depth and the color of the solution to be examined is matched with it by raising or lowering cups. When the color is of the same intensity as the standard the depth of the solution is read. The amounts of the colored substance in solution are inversely proportional to the depths of the columns of fluid. Thus if the standard is set at 10 mm. and the solution under examination has the same color density at 20 mm. the latter has just one-half the concentration of the standard.

A large number of other colorimeters have been devised and may be used in place of the Duboscq. Most of these though less expensive than this instrument are also less accurate. The Hellige colorimeter has been recommended, particularly for clinical determinations by Myers and Fine. A simple colorimeter, costing only about one dollar, has been devised and used with considerable success by Peebles and Lewis. It is claimed to compare favorably in accuracy with other colorimeters and to be applicable to clinical and student use. Another accurate colorimeter has been developed by Bock and Benedict in which the place of costly and difficultly obtainable prisms is taken by mirrors. Kober has devised a combined colorimeter and nephelometer which may be obtained in this country. For merely approximate determinations the color comparisons may be made directly with a series of colored standards of varying strengths made up in exactly similar test-tubes or small flasks.

UREA

Urease Methods

Principle.—These methods depend upon the principle that the enzyme urease is able, at ordinary temperature, to transform urea, quickly and completely, into ammonium carbonate. Takeuchi in 1909 discovered the presence of this enzyme in the soja or soy bean. The application of this enzyme to the determi-

nation of urea in urine, blood, etc., was first proposed by Marshall, whose methods have been modified by Van Slyke and Cullen. These latter investigators prepared a permanent preparation of the enzyme, in a water-soluble form, the use of which makes more convenient the rapid and accurate determination of urea in urine, blood and other biological fluids.

The urease method is probably the most satisfactory of all methods for the determination of urea. Other nitrogenous constituents such as allantoin are not decomposed by urease. The method involves no carefully regulated heating procedures, and is applicable to diabetic urines.

The procedure for the determination in urine consists in treating the urine sample with urease, adding an alkali and aerating the liberated ammonia into fiftieth-normal acid, and titrating the excess of acid with fiftieth-normal alkali.

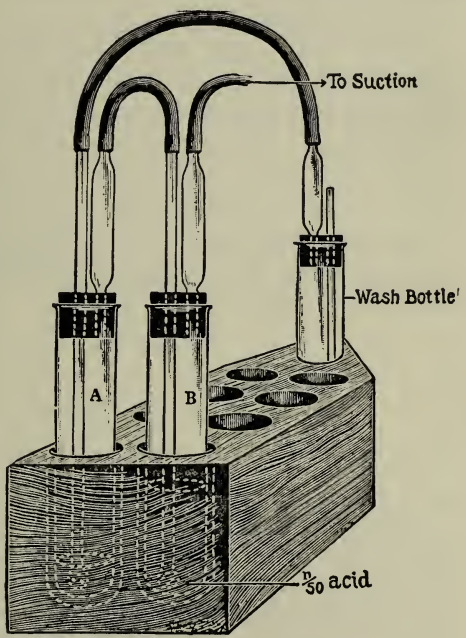

FIG. 1.—Van Slyke and Cullen Apparatus.

Procedure of Van Slyke and Cullen.[1]—Dilute 5 c.c. of urine to 50 c.c. with ammonia-free water. Measure 5 c.c. of the diluted urine into Tube "A" of the Van Slyke and Cullen apparatus (Fig. 1) add 1 drop of caprylic alcohol (to prevent frothing), and 1 c.c. of enzyme solution. Close "A" with stopper and let the tube stand fifteen minutes for the enzyme to act. Measure into Tube "B" 25 c.c. of N/50 HCl or H_2SO_4. Add 1 drop of caprylic alcohol and 1 drop of a 1 per cent alizarin solution, as indicator. Connect Tubes "A" and "B" so that the current of air passes from the urine to the acid. Let the air be

[1] Van Slyke and Cullen: Jour. Biol. Chem., 1914, 19, 211.

drawn through a tube containing strong sulphuric acid before it is admitted to Tube " A " so that all the ammonia of the air may be absorbed. At the end of fifteen minutes aspirate for about one-half minute to remove any ammonia present in the free condition in " A." After this aspiration, open " A " and introduce 5 c.c. of saturated potassium carbonate. Close " A " at once and aspirate until all the ammonia has been removed from " A " and carried over into the acid in " B." The time needed for the aspiration varies for different pumps from five to thirty minutes, and should be determined by trial for the particular apparatus used. At the end of the time needed for the aeration, the pump is disconnected (care being taken to avoid back suction) and the excess acid in " B " is titrated by means of fiftieth-normal alkali.

Calculation.—The number of cubic centimeters of fiftieth-normal acid neutralized is multiplied by the factor 0.056 to give the number of grams of urea plus ammonia-nitrogen in 100 c.c. of the urine. The ammonia alone may be determined at the same time as the ammonia plus urea, using the same technic except that 5 c.c. of the undiluted urine, no urease, and the factor 0.0056 are used for the determination of ammonia alone. The ammonia tubes are run in the same series as these for the urea determination, using the same air current for all.

UREA

Marshall's Urease Method [1]

Principle.—This is a simple clinical method for the determination of urea in urine. It differs from the preceding method in that instead of aspirating off the ammonia formed from the urea by the action of the urease, it is titrated directly in the urine mixture, thus simplifying the procedure. The method is nearly as accurate as the preceding for normal urine the error being only about 2 per cent which is very satisfactory for a rapid clinical procedure. For diabetic urines the aeration procedure should be used as such urines contain substances which render the titration inaccurate.

[1] Marshall: Jour. Biol. Chem., 1913, 14, 283.

Procedure.—Two 5 c.c. portions of the urine are measured into flasks of 200 to 300 c.c. capacity and diluted with distilled water to about 100 to 125 c.c. One c.c. of a 10 per cent solution of urease is added to one flask, a few drops of toluene to each and the solution allowed to remain, well stoppered, at room temperature over night (or five hours). The fluid in each flask is titrated to a distinct pink color with N/10 hydrochloric acid using methyl orange as an indicator. A few cubic centimeters of the enzyme solution used should also be titrated to determine the amount of N/10 hydrochloric acid required to neutralize 1 c.c.

Calculation.—The amount of hydrochloric acid required for the contents of the flask containing the urine and enzyme solution, less the amount used for 5 c.c. of urine alone and that previously determined for 1 c.c. of enzyme solution, corresponds to the urea originally present in the sample of urine. Since 1 c.c. of N/10 HCl is equivalent to 3 mg. of urea, the number of cubic centimeters required, multiplied by 0.6 gives the value of urea expressed in grams per liter of urine.

DETERMINATION OF UREA IN URINE BY DIRECT NESSLERIZATION [1]

Principle.—By using a urease preparation sufficiently free from nitrogenous materials the urea nitrogen can be Nesslerized without treatment by charcoal for purposes of purification.

Procedure.—Wash 3 gms. of permutit in a flask, once with 2 per cent acetic acid, and twice with water; add 5 gms. of fine jack bean meal and 100 c.c. of 30 per cent alcohol. Shake gently but continuously for ten to fifteen minutes, and filter. The filtrate contains practically the whole of the urease and extremely little of other materials. Add 1 c.c. of this urease solution to 1 c.c. of diluted urine (dilution usually 1:10) in a test-tube, and digest in a beaker of warm water (40° to 55° C.) for five minutes, or at room temperature for fifteen minutes. It is preferable, but not necessary, to add a drop of a suitable phosphate solution to the mixed contents in the test-tube at the beginning of the digestion. The buffer mixture is particularly desirable if the digestion is to be made at room temperature. At the end of the

[1] Folin and Youngburg: Jour. Biol. Chem., 1919, 38, 111.

digestion period transfer the contents of the test-tube to a 200 c.c. volumetric flask, diluting to a volume of 150 c.c. Add 1 mg. of nitrogen in the form of ammonium sulphate to another volumetric flask. To this standard add 1 c.c. of the urease solution and dilute to about 150 c.c. Then add 20 c.c. of the Nessler solution prepared according to the directions given on page 87. Dilute to volume and make the color comparison. The ammonia nitrogen, is, of course, included in the figure obtained.

Remarks.—This determination may be made in urines very rich in albumin. Because of the extremely low nitrogen content of the urease preparation, it is not really essential that the urease should also be added to the standard ammonia solution, but it is added simply as a precaution against the possible occurrence of less pure urease preparations.

AMMONIA

Folin's Method[1]

Principle.—The ammonia of the urine is set free by the addition of an alkali and this ammonia is then carried over by an air current into a flask containing a measured amount of standard acid. The excess is then titrated. The necessity for distillation is avoided.

Procedure.—Place 25 c.c. of urine in an aerometer cylinder, 30 to 40 cm. in height, add about 1 gm. of dry sodium carbonate and introduce some crude petroleum to prevent foaming. Insert into the neck of the cylinder a rubber stopper provided with two perforations, into each of which passes a glass tube, one of which reaches below the surface of the liquid. The shorter tube (10 cm. in length) is connected with a calcium chloride tube filled with cotton, and this tube is in turn joined to a glass tube extending to the bottom of a 500 c.c. wide-mouthed flask which is intended to absorb the ammonia and for this purpose should contain 20 c.c. of N/10 sulphuric acid, 200 c.c. of ammonia-free distilled water and a few drops of an indicator (alizarin red or Congo red). To insure the complete absorption of the ammonia the absorption flask is provided with a Folin improved absorption tube which is very effective in causing the air passing from the cylinder to

[1] Folin: Am. Jour. Physiol., 1915, 13, 45.

come into intimate contact with the acid in the absorption flask. In order to exclude any error due to the presence of ammonia in the air a similar absorption apparatus to the one just described is attached to the other side of the aerometer cylinder, thus insuring the passage of ammonia-free air into the cylinder. With an ordinary filter pump and good water pressure the last trace of ammonia should be removed from the cylinder in about one and one-half hours. The number of cubic centimeters of the N/10 sulphuric acid neutralized by the ammonia of the urine may be determined by direct titration with N/10 sodium hydroxide.

Steele[1] has suggested a modification for use on urines containing triple phosphate sediments. In this modification 0.5 to 1.0 gm. of NaOH and about 15 gms. of NaCl are substituted for the Na_2CO_3 of the Folin method. The use of sodium hydroxide and chloride instead of carbonate has also been recommended by other workers as a general procedure, inasmuch as triple phosphate crystals are almost always formed on adding sodium carbonate and these are decomposed with some difficulty by sodium carbonate but readily by the hydroxide. It has not been shown that the use of sodium hydroxide in this manner brings about the decomposition of any other urinary nitrogen compounds.

Calculation.—Subtract the number of cubic centimeters of N/10 sodium hydroxide used in the titration from the number of cubic centimeters of N/10 sulphuric acid taken. The remainder is the number of cubic centimeters of N/10 sulphuric acid neutralized by the NH_3 of the urine. One c.c. of N/10 sulphuric acid is equivalent to 0.0017 gm. of NH_3. Therefore if y represents the volume of urine used in the determination and y' the number of cubic centimeters of N/10 sulphuric acid neutralized by the NH_3 of the urine, we have the following proportion:

$y : 100 :: y' \times 0.0017 : x$ (percentage of NH_3 in the urine examined).

Calculate the quantity of NH_3 in the twenty-four-hour urine specimen.

[1] Steele: Jour. Biol. Chem., 1910, 8, 365.

AMMONIA

Microchemical Method of Folin and MacCallum[1]

Principle.—This method is a combination of the aeration procedure for ammonia with its colorimetric determination by means of Nessler-Winkler solution. It gives satisfactory results, but is probably not as accurate as the regular Folin procedure where the amount of substance for analysis is not limited.

Procedure.—By means of Ostwald pipettes introduce 1 to 5 c.c. of urine into a Jena test-tube (20 to 25 mm. by 200 mm.) and add to the urine a few drops of a solution containing 10 per cent of potassium carbonate and 15 per cent of potassium oxalate. To prevent foaming add a few drops of kerosene or heavy, crude machine oil. Pass a strong air current (see page 42) through the mixture until the ammonia has been entirely removed. Collect the ammonia in a 100 c.c. volumetric flask containing about 20 c.c. of ammonia-free water and 2 c.c. of N/10 acid.

Nesslerize as described in the method for total nitrogen, page 36, and compare with 1 mg. of nitrogen obtained from a standard ammonium sulphate solution and similarly Nesslerized.

It has been noted that a trace of something capable of giving a color with the Nessler-Winkler solution continues to come along after all the ammonia has been removed from the urine. The nature of this substance has not yet been determined. In actual determinations by this method, the influence of this unknown substance, because of the small volume of urine used, is entirely negligible.

CREATININE

Folin's Colorimetric Method[2]

Principle.—This method is based upon the characteristic property possessed by creatinine of yielding a certain definite color-reaction in the presence of picric acid in alkaline solution.

Procedure.—Place 10 c.c. of urine in a 500 c.c. volumetric flask, add 15 c.c. of a saturated solution of picric acid and 5 c.c. of a 10 per cent solution of sodium hydroxide, shake thoroughly

[1] Folin and MacCallum: Jour. Biol. Chem., 1912, 11, 523.

[2] Folin: Am. Jour. Physiol., 1905, 13, 45.

and allow the mixture to stand for five minutes. During this interval pour a little N/2 potassium bichromate solution into each of the two cylinders of the colorimeter (Duboscq) and carefully adjust the depth of the solution in one of the cylinders to the 8 mm. mark. A few preliminary colorimetric readings may now be made with the solution in the other cylinder, in order to insure greater accuracy in the subsequent examination of the solution of unknown strength. Obviously the two solutions of potassium bichromate are identical in color and in their examination no two readings should differ more than 0.1 to 0.2 mm. from the true value (8 mm.). Four or more readings should be made in each case and an average taken of all of them exclusive of the first reading, which is apt to be less accurate than the succeeding readings. In time as one becomes proficient in the technic it is perfectly safe to take the average of the first two readings.

At the end of the five-minute interval already mentioned, the contents of the 500 c.c. flask are diluted to the 500 c.c. mark, the bichromate solution is thoroughly rinsed out of one of the cylinders and replaced with the solution thus prepared and a number of colorimetric readings are immediately made.

Ordinarily 10 c.c. of urine is used in the determination by this method, but if the content of creatinine is above 15 mg. or below 5 mg. the determination should be repeated with a volume of urine selected according to the content of creatinine. This variation in the volume of urine according to the content of creatinine is quite essential, since the method loses in accuracy when more than 15 mg. or less than 5 mg. of creatinine is present in the solution of unknown strength.

Calculation.—By experiment it has been determined that 10 mg. of pure creatinine, when brought into solution and diluted to 500 c.c. as explained in the above method, yields a mixture 8.1 mm. of which possesses the same colorimetric value as 8 mm. of a N/2 solution of potassium bichromate. Bearing this in mind the computation is readily made by means of the following proportion in which y represents the number of millimeters of the solution of unknown strength equivalent to the 8 mm. of the potassium bichromate solution:

$y : 8.1 :: 10 : x$ (mg. of creatinine in the quantity of urine used).

This proportion may be used for the calculation no matter what volume of urine (5, 10, or 15 c.c.) is used in the determination. The 10 represents 10 mg. of creatinine which gives a color equal to 3.1 mm., whether dissolved in 5, 10, or 15 c.c. of fluid.

Calculate the quantity of creatinine in the twenty-four hour urine specimen.

Use of Pure Creatinine Standards

Instead of using as a standard a potassium dichromate solution as above indicated, a solution of pure creatinine is to be recommended. By using this certain arbitrary factors are eliminated and the method becomes of more general applicability. The standard need not be set at a definite mark as is necessary in the case of dichromate and temperature and time have less influence on the accuracy of the results. A stock solution of pure creatinine is made by dissolving 1 gm. of the substance in sufficient N/10 HCl to make a liter. This solution contains 1 mg. of creatinine per cubic centimeter. In carrying out the determination treat 10 c.c. of the stock solution in the same way and at the same time as the 10 c.c. sample of urine. Compare in the colorimeter. The calculation is simple. The reading of the standard divided by the reading of the urine gives directly the number of milligrams of creatinine per cubic centimeter of urine.

CREATININE

Folin's Microchemical Modification[1]

Principle.—The principle is the same as that of the original colorimetric method. This procedure is to be recommended particularly where only small amounts of material are available.

Procedure.—One c.c. of the standard creatinine (see above) solution (1 mg. per c.c.) is measured into a 100 c.c. volumetric flask and 1 c.c. of urine into another; 20 c.c. of saturated picric acid solution (measured with a cylinder) are added to each and then 1.5 c.c. of a 10 per cent solution of sodium hydroxide. At the end of ten minutes the flasks are filled up to the mark with

[1] Folin: Jour. Biol. Chem., 1914, 17, 469.

tap water and the color of the unknown is determined. The reading of the standard divided by the reading of the unknown gives directly the number of milligrams of creatinine in the amount of urine taken for analysis.

CREATININE

Shaffer's Modification for the Determination of Creatinine in Very Dilute Solutions [1]

The regular Folin procedure is not accurate when applied to urines containing less than 20 mg. of creatinine per 100 c.c. By a slight modification it becomes applicable to creatinine solutions containing as little as 1 mg. or less per 100 c.c.

Procedure.—To the solution under examination add an equal volume of saturated picric acid solution and one-tenth this volume of 10 per cent sodium hydroxide solution. After standing six to ten minutes the liquid is diluted to a definite volume depending upon the intensity of the color developed. With very dilute solutions one may add solid picric acid equivalent to half saturation (0.6 per cent) and when dissolved, one-twentieth the volume of sodium hydroxide. Provided the creatinine solution itself has not sufficient color to interfere, the results by this method appear to be as accurate as the original procedure. The colorimetric readings and calculations are made in the same way as in the preceding methods.

CREATINE

Folin-Benedict Method [2]

Principle.—Creatine on boiling with acid is transformed into creatinine. By determining the content of creatinine before and after the acid treatment we are able to calculate the amount of creatine originally present in the urine. The Folin colorimetric method is used for determining the creatinine in both cases. The method is not applicable to diabetic urines.

Procedure.—Introduce into a small flask or beaker 10 c.c. of the urine to be examined. (If 10 c.c. contains more than 12 or

[1] Shaffer: Jour. Biol. Chem., 1914, 18, 525.

[2] Benedict: Jour. Biol. Chem., 1914, 18, 191.

less than 7 mg. of total creatinine use a correspondingly smaller or larger volume of urine.) Add from 10 to 20 c.c. of normal HCl, and a pinch or two of powdered or granulated lead. Boil the mixture over a free flame as slowly or as rapidly as may be desired, until very nearly down to dryness, when the heating should be continued to dryness either on the water-bath or very easily by simply holding the vessel in the hand and heating carefully for a moment or two. Let the residue stand on the water-bath for a few minutes until most of the excess of hydrochloric acid gas has been expelled, after which dissolve it in about 10 c.c. of hot water and rinse the solution quantitatively through a plug of cotton or glass wool (to remove all metallic lead) into a 500 c.c. volumetric flask. Add 20 to 25 c.c. of a saturated picric acid solution and about 7 to 8 c.c. of a 10 per cent NaOH solution, which contains 5 per cent of Rochelle salt. At the end of five minutes fill to the mark with water and read in the colorimeter just as in the case of creatinine.

Calculation.—Calculate the creatinine content of the solution in the same manner as given under Creatinine. From the value thus obtained subtract the value for the creatinine content of the urine before dehydration. The difference will be the creatine content of the original urine in terms of creatinine.

CREATINE

Microchemical Modification of Folin [1]

Principle.—By greatly diluting the urine the time required for the conversion of creatine to creatinine is decreased, and picric acid can be substituted for mineral acid.

Procedure.—Enough urine to give 0.7 to 1.5 mg. of creatinine is measured into a weighed Erlenmeyer Jena flask (capacity 200 c.c.); 20 c.c. of saturated picric acid solution, about 130 c.c. of water, and a few very small pebbles to promote even boiling are added and the mixture is gently boiled, preferably over a micro-burner for about one hour. At the end of this time the heat is increased and the solution is boiled down to rather less than 20 c.c. The flask is transferred to the scales and enough water is added to make the total solution equal to 20 to 25 gms.

[1] Folin: Jour. Biol. Chem., 1914, 17, 469.

The solution is cooled in running water, 1.5 c.c. of 10 per cent sodium hydroxide are added, and the total creatinine is determined as in the preformed creatinine determination using 1 mg. of creatinine as a standard. The method has been found to give good results in the presence of glucose and other sugars.

Morris has suggested that in the case of diabetic urines the total creatinine be determined after precipitation of the creatine and creatinine with picric acid. The method is not recommended as a regular procedure.

URIC ACID

Folin-Shaffer Method [1]

Principle.—The uric acid is precipitated as ammonium urate by the addition of ammonia, the precipitate filtered off, washed and titrated with potassium permanganate. A preliminary treatment with an ammonium sulphate-uranium acetate solution is for the purpose of removing interfering organic substances. The method gives accurate results.

Procedure.—Introduce 100 c.c. of urine into an Erlenmeyer flask, add 25 c.c. of the Folin-Shaffer reagent and after shaking the flask to thoroughly mix the fluids allow the mixture to stand, with or without further stirring, until the precipitate has settled (five to ten minutes). Filter, transfer 100 c.c. of the filtrate to a 200 c.c. Erlenmeyer flask, and 5 c.c. of concentrated ammonium hydroxide and allow the mixture to stand for twenty-four hours. Transfer the precipitated ammonium urate quantitatively to a filter paper, using 10 per cent ammonium sulphate to remove the final traces of the urate from the flask. Wash the precipitate approximately free from chlorides by means of 10 per cent ammonium sulphate solution, remove the paper from the funnel, open it, and by means of hot water rinse the precipitate back through the funnel into the flask in which the urate was originally precipitated. The volume of fluid at this point should be about 100 c.c. Cool the solution to room temperature, add 15 c.c. of concentrated sulphuric acid and titrate at once with N/20 potassium permanganate, $K_2Mn_2O_8$, solution. The first tinge of pink color which extends throughout the fluid after the addi-

[1] Folin and Shaffer: Zeit. physiol. Chem., 1901, 32, 552.

tion of two drops of the permanganate solution, while stirring with a glass rod, should be taken as the end-reaction. Take the burette reading and compute the percentage of uric acid present in the urine under examination.

Calculation.—Each cubic centimeter of N/20 potassium permanganate solution is equivalent to 3.75 mg. (0.00375 gm.) of uric acid.

The 100 c.c. from which the ammonium urate was precipitated is equivalent to only four-fifths of the 100 c.c. of urine originally taken; therefore we must take five-fourths of the burette reading in order to ascertain the number of cubic centimeters of the permanganate solution required to titrate 100 c.c. of the original urine to the correct end point. If y represents the number of cubic centimeters of the permanganate solution required, we may make the following calculation:

$$y \times 0.00375 = \text{weight of uric acid in 100 c.c. of urine.}$$

Because of the solubility of the ammonium urate a correction of 3 mg. should be added to the final result.

Calculate the quantity of uric acid in the twenty-four hour urine specimen.

URIC ACID

Microchemical Colorimetric Method

Benedict and Hitchcock Modification of the Folin-Macallum-Denis Proecdure.[1] *Principle.*—The principle of the method depends upon the fact, first noted by Folin and Macallum and further investigated by Folin and Denis, that uric acid gives, with phosphotungstic acid and alkali, a deep blue color the depth of which is proprotional to the amount of uric acid present. Since certain other substances present in urine produce a similar blue color with the phosphotungstic acid, it is necessary to separate the uric acid from them. This is accomplished by precipitation as the silver salt. The silver urate is subsequently dissolved and treated with the uric acid reagent.

[1] Folin and Macallum: Jour. Biol. Chem., 1912, 12, 363. Folin and Denis: Jour. Biol. Chem., 1913, 14, 95; *ibid.*, 1913, 13, 469. Benedict and Hitchcock: Jour. Biol. Chem., 1915, 20, 619; Benedict: *ibid.*, 1915, 20, 629.

Benedict and Hitchcock have examined the method of Folin and Denis and have suggested a number of important modifications.

Procedure.—Measure such an amount of urine as will contain from 0.7 to 1.3 mg. of uric acid (2 to 4 c.c. is usually the correct amount) into a centrifuge tube, dilute with water to about 5 c.c. and add 15 to 20 drops of an ammoniacal silver magnesium solution. Mix the contents of the tube with a small stirring rod and centrifuge the tube for one or two minutes. Pour off the supernatant liquid, as completely as possible, by inverting the tube, allowing it to drain a moment, and then touching the inside of the lip of the tube with a towel or piece of filter paper. Add to the residue in the tube 2 drops of 5 per cent solution of potassium cyanide to dissolve the silver urate, stir the mixture thoroughly with a thin rod, for half a minute, add a few drops (0.5 to 1.0 c.c.) of water, and stir again. Two c.c. of the uric acid reagent are added and the mixture stirred again, after which add 10 c.c. of 20 per cent sodium carbonate solution, transfer quantitatively to a 50 c.c. flask, and at the end of about one-half minute, dilute to mark. Compare this solution in the Duboscq colorimeter with a simultaneously prepared solution obtained by treating 5 c.c. of the standard uric acid solution, contained in a 50 c.c. flask, with 2 drops of the potassium cyanide solution, 2 c.c. of the uric acid reagent, 10 c.c. of 20 per cent sodium carbonate solution, diluting to the mark at the end of about one-half minute. The standard solution is best set at a height of 15 mm. in the colorimeter.

Calculation.—The reading of the standard divided by the reading of the urine gives the number of milligrams of uric acid in the amount of sample taken.

COLORIMETRIC METHOD FOR DETERMINATION OF URIC ACID IN URINE

Folin and Wu[1]

Principle.—Uric acid is precipitated as the urate by means of silver lactate. Silver urate is then dissolved in an alkaline solution of sodium cyanide, and a uric acid reagent is then added. This

[1] Folin and Wu: Jour. Biol. Chem., 1919, 38, 459.

reagent gives to uric acid an intense color, the depth of which is compared with the standard solution of uric acid also treated with this reagent.

Solutions needed.—1. *Standard uric acid solution.* Before starting to prepare the uric acid solution a 20 per cent filtered solution of sodium sulphite should be available. Dissolve 1 gm. of uric acid in a 125 to 150 c.c. of 0.4 per cent lithium carbonate solution and dilute to a volume of 500 c.c. Transfer 50 c.c. corresponding to 100 mg. of uric acid, to each of a series of volumetric liter flasks. Add about 300 c.c. of water and then add 500 c.c. of clear 20 per cent sodium sulphite solution, mix, dilute to volume, and mix thoroughly. Fill a series of 200 c.c. bottles, and stopper very tightly. The reason why a series of small bottles is used as containers is, of course, to reduce the absorption of oxygen from the air.

2. *A 10 per cent sodium sulphite solution*, kept like the uric acid solution, in small tightly stoppered bottles.

3. *A 5 per cent sodium cyanide solution.*

4. *A solution containing 5 per cent of silver lactate* and 5 per cent of lactic acid.

5. *The uric acid reagent of Folin and Denis.* To prepare this boil 100 gms. of sodium tungstate with 80 c.c. of phosphoric acid (85 per cent) and 700 c.c. of water, for not less than two hours, and dilute to 1 liter.

Procedure.—Transfer from 1 to 3 c.c. of urine to a 15 c.c. centrifuge tube and mix with enough water to make a volume of about 6 c.c. Add 5 c.c. of the acid silver lactate solution and stir with a very fine glass rod (diameter 1 to 2 mm.), rinse off the rod with a few drops of water and centrifuge. If enough silver solution has been added the precipitate settles very quickly. Add a drop of silver lactate solution so as to be certain that an excess is present; if a precipitate forms add more (2 c.c.) of the silver lactate solution and centrifuge again. In point of fact the first 5 c.c. addition of silver lactate is usually sufficient but it is not safe to omit the test. Pour off the clear supernatant liquid as completely as possible.

To the precipitate in the centrifuge tube add, from a burette, 4 c.c. of 5 per cent sodium cyanide solution, and stir until a perfectly clear solution is obtained. Pour the contents into a 100 c.c. volumetric flask and rinse the tube and stirring rod, using for this

purpose about 15 to 25 c.c. of water. Add 5 c.c. of 10 per cent sodium sulphite solution (to balance the sulphite in the standard uric acid solution). Dilute to a volume of about 50 c.c.

Transfer to another 100 c.c. flask 5 c.c. of the standard uric acid sulphite solution, containing 0.5 mg. uric acid. Add 4 c.c. of the cyanide solution *from a burette* and dilute to about 50 c.c. Then add 20 c.c. of saturated sodium carbonate solution to each flask, mix, and finally add *with shaking*, 2 c.c. of the uric acid reagent. Let stand for three to five minutes, fill to the mark, mix, and make the color comparison in the usual manner, never omitting to first read the standard against itself. Artificial light (with "daylite" glass) is better than daylight for this color comparison.

Calculation.—With the standard set at 20 mm., 10 divided by the reading of the unknown (in mm.) gives the amount of uric acid (in mg.) in the volume of urine taken.

Remarks.—The discarded blue solution should be poured as directly as possible into the drain of sinks on account of the cyanide.

PURINE BASES

Krüger and Schmidt's Method[1]

Principle.—This method serves for the determination of both uric acid and the purine bases. The principle involved is the precipitation of both the uric acid and the purine bases in combination with copper oxide and the subsequent decomposition of this precipitate by means of sodium sulphide. The uric acid is then precipitated by means of hydrochloric acid and the purine bases are separated from the filtrate in the form of their copper or silver compounds. The nitrogen content of the precipitates of uric acid and purine bases is then determined by means of the Kjeldahl method (see page 33) and the corresponding values for uric acid and purine bases calculated.

Procedure.—To 400 c.c. of albumin-free urine in a liter flask, add 24 gms. of sodium acetate, 40 c.c. of a solution of sodium bisulphite, and heat the mixture to boiling. Add 40 to 80 c.c.

[1] Krüger and Schmidt: Zeit. f. physiol. Chem., 1905, 45, 1.

of a 10 per cent solution of copper sulphate and maintain the temperature of the mixture at the boiling-point for at least three minutes. Filter off the flocculent precipitate, wash it with hot water until the wash water is colorless, and return the washed precipitate to the flask by puncturing the tip of the filter paper and washing the precipitate through by means of hot water. Add water until the volume in the flask is approximately 200 c.c., heat the mixture to boiling and decompose the precipitate of copper oxide by the addition of 30 c.c. of sodium sulphide solution. After decomposition is complete, the mixture should be acidified with acetic acid and heated to boiling until the separating sulphur collects in a mass. Filter the hot fluid by means of a filter-pump, wash with hot water, add 10 c.c. of 10 per cent hydrochloric acid and evaporate the filtrate in a porcelain dish until the total volume has been reduced to about 10 c.c. Permit this residue to stand about two hours to allow for the separation of the uric acid, leaving the purine bases in solution. Filter off the precipitate of uric acid, using a small filter paper, and wash the uric acid, with water made acid with sulphuric acid, until the total volume of the original filtrate and the wash water aggregates 75 c.c. Determine the nitrogen content of the precipitate by means of the Kjeldahl method (see page 33), and calculate the uric acid equivalent.

Render the filtrate from the uric acid crystals alkaline with sodium hydroxide, add acetic acid until faintly acid and heat to 70° C. Now add 1 c.c. of a 10 per cent solution of acetic acid and 10 c.c. of a suspension of manganese dioxide to oxidize the traces of uric acid which remain in the solution. Agitate the mixture for one minute, add 10 c.c. of the sodium bisulphite solution and 5 c.c. of a 10 per cent solution of copper sulphate and heat the mixture to boiling for three minutes. Filter off the precipitate, wash it with hot water, and determine its nitrogen content by means of the Kjeldahl method (see page 33). Inasmuch as the composition and proportion of the purine bases present in urine is variable, no factor can be applied. The result as regards these bases must therefore be expressed in terms of nitrogen.

Benedict and Saiki report cases in which the total purine nitrogen by this method was less than the uric-acid nitrogen as determined by the Folin-Shaffer method. The inaccuracy was found to lie in the Kruger and Schmidt method. To obviate

this they advise the addition of 20 c.c. of glacial acetic acid for each 300 c.c. of urine employed, the acid being added before the first precipitation.

AMINO-ACID NITROGEN

Henriques-Sörensen Formol Titration Method[1]

Principle.—A solution containing amino-acids is nearly neutral in reaction. If formaldehyde be added, however, the following reaction takes place with the formation of methylene derivatives which are more strongly acid in reaction due to the destruction of the basic properties of the amino groups. The carboxyl groups may then be titrated, using phenolphthalein as an indicator.

$$\begin{matrix} R.CH.NH_2 \\ | \\ COOH \end{matrix} + CH_2O = \begin{matrix} R-CH-N:CH_2 \\ | \\ COOH \end{matrix} + H_2O.$$

The acidity as shown by the titration is a measure of the amount of amino-acid nitrogen present. Ammonia likewise reacts with formaldehyde in a similar manner as is shown in the following equation:

$$4NH_4Cl + 6CH_2O = N_4(CH_2)_6 + 6H_6O + 4HCl.$$

Hence the formol titration in the presence of ammonia gives results which include both amino-acid and ammonia nitrogen. Ammonia may be determined and a correction applied, or the ammonia may be removed by means of phosphotungstic acid. Phosphates also interfere by obscuring the end-point and are removed by the addition of barium salts.

It must be borne in mind that polypeptides and still more complex protein derivatives likewise react with formol to a certain degree so that the results do not strictly represent "amino-acid nitrogen."

The method is, with some modifications involving the preparation of the solution to be titrated, applicable in the determination of amino-acids in any medium, e.g., urine, protein digests,

[1] Henriques and Sörensen: Zeit. physiol. Chem., 1909, 64, 120.

etc. When poorly dissociated acids, e.g., some fatty acids, are present, these will in part be included in the result and lead to values which are too high. Certain of the amino-acids when present in large amounts will give erroneous results, but in the ordinary urine or digest these errors are either negligible or compensate each other. In the titration of colored solutions the control solution which is necessary in this method must be colored to correspond with the color of the unknown solution.

Procedure.—The determination of the amino-acids is carried out as follows: The solution to be analyzed, if carbonates, phosphates and ammonia are absent, is made neutral to litmus (paper) and the solution titrated with formaldehyde. In case carbonates, phosphates or ammonia are present a preliminary treatment is necessary which will vary according to the quantity of ammonia present.

(*a*) *For Small Amounts of Ammonia.*—Applicable to most urines. Fifty c.c. of the material under examination is pipetted into a 100 c.c. measuring flask and 1 c.c. phenolphthalein solution and 2 gms. of solid barium chloride are added; the whole is shaken, to saturate the solution with barium chloride; saturated barium hydroxide solution is added until the red color of the phenolphthalein develops and then an excess of 5 c.c. is added. The flask is filled to the graduation mark with water, shaken and permitted to stand for fifteen minutes, after which it is filtered through a dry filter. Eighty c.c. of the clear red filtrate (which corresponds to 40 c.c. of the liquid under examination) are placed in a 100 c.c. measuring flask, neutralized to litmus and diluted to 100 c.c. with freshly boiled water. Equal portions of this solution, 40 c.c. (equivalent to 16 c.c. of the original solution), may be taken for analysis, one for the formol titration and the other for the determination of ammonia nitrogen.

(*b*) *For Large Amounts of Ammonia.*—After the treatment with phenolphthalein, barium chloride, and barium hydroxide, and the solution has been diluted to 100 c.c. as in (*a*) above, the ammonia is distilled off, *in vacuo*.

In case the solution is deeply colored, as in protein digests, it may be necessary to decolorize before the titration is attempted.

Final Titration.—For the final titration a volume of from 20 to 40 c.c. which contains approximately 0.0125 gm. of nitrogen is the most desirable. A control solution is run composed

of an equal volume of boiled distilled water and 20 c.c. of the formaldehyde mixture. This control solution is colored so that its tint matches that of the solution to be titrated.

To this control is added about half the volume of N/5 alkali which will be used in the titration of the solution under investigation and it is then titrated with N/5 acid to a faint red (first stage).

An additional drop of N/5 alkali is added, which imparts a distinct red to the solution (second stage).

The solution to be analyzed is now titrated to the color produced in the second stage of the control. The formaldehyde mixture is now added; 10 c.c. for each 20 c.c. of the solution, and the mixture again titrated to the second stage with N/5 alkali.

Two drops of the N/5 alkali are now added to the control solution which assumes a deep red color (third stage). Fifth normal alkali is now added to the solution under examination until it assumes a color corresponding to the third stage of the control. This completes the titration.

Calculation.—The calculations are similar to those which pertain to any acidimetry procedure. Each cubic centimeter of an N/5 alkali or acid solution is equivalent to 0.0028 gm. of nitrogen. An example will illustrate the procedure: 40 c.c. of solution (16 c.c. of urine) required 5.10 c.c. N/5 NaOH; control, 0.10 c.c. N/5 NaOH; total required for amino-acids 5.00 c.c. equivalent to 0.014 gm. of nitrogen. Ammonia nitrogen in 16 c.c. of urine 0.007 gm. N. Then $0.014 - 0.007 = 0.007$ gm. amino-acid nitrogen in 16 c.c. of urine.

AMINO-ACID NITROGEN

Van Slyke's Method for Total Amino-Acid Nitrogen[1]

Take 25 c.c. of urine and mix with 1 c.c. of concentrated sulphuric acid and heat in an autoclave at 180° (oil bath temperature) for one and one-half hours. Transfer to a 50 c.c. flask and add 2 gms. powdered calcium hydroxide. Shake thoroughly, make up to 50 c.c. and filter through a dry folded filter. Transfer 20 c.c. of the filtrate to a Jena glass evaporating dish and concentrate to dryness on the water-bath. This requires about half

[1] Van Slyke: Jour. Biol. Chem., 1913, 16, 125.

an hour. The residue is moistened with 1 c.c. of 50 per cent acetic acid to bring the calcium hydroxide and carbonate into solution, and is then washed into a 10 c.c. flask and filled up to the mark. One can use the entire solution for determination of the amino-nitrogen in the large amino-apparatus, or use 2 c.c. portions for the micro-apparatus.

The length of time which the nitrous acid solution should be shaken in order to drive off all the amino-nitrogen depends somewhat on the temperature. When the latter is 15 to 20° the time should be five to four minutes; for 20 to 25° it is three minutes; for 25 to 30°, two and a half to two minutes. It is preferable that the solution should be shaken vigorously with a motor and the time kept down to these limits, for the sake not only of rapidity but of accuracy.

Van Slyke's Method for Free Amino-Acid Nitrogen

To 25 c.c. of urine in a 50 c.c. flask add urease solution and allow to stand for one and one-half times the interval which has been found necessary to affect the maximum decomposition of urea, as observed by titration of the ammonia. The last traces of urea are decomposed. At the end of the digestion period 10 c.c. of a 10 per cent suspension of calcium hydroxide are added, the mixture shaken and made up to 50 c.c. Then filter, evaporate, and complete the determination according to the method outlined under total amino-acid nitrogen, above.

HIPPURIC ACID

Method of Folin and Flanders[1]

Principle.—The hippuric acid is hydrolyzed to benzoic acid in alkaline solution and then the solution is boiled with strong nitric acid to remove pigments and emulsifying substances. The benzoic acid is extracted with chloroform and titrated with sodium ethylate.

Procedure.—Measure 100 c.c. of urine into a porcelain evaporating dish by means of a pipette. Add 10 c.c. of 5 per cent NaOH and evaporate to dryness on the steam-bath. Transfer the resi-

[1] Folin and Flanders: Jour. Biol. Chem., 1912, 11, 257.

due to a 500 c.c. Kjeldahl flask by means of 25 c.c. of water and 25 c.c. of concentrated HNO_3. Add 0.2 gm. of copper nitrate, a couple of pebbles or glass pearls and boil very gently for four and one-half hours over a micro-burner. Fit the necks of the flasks with condensers of the Hopkins type made from large test-tubes fitted with two-hole rubber stoppers, the inlet tubes extending near the bottom of the test-tubes while the outlet tube is shorter. These condensers should fit rather loosely. A good current of water flowing through the condensers prevents loss of benzoic acid or change in concentration of the nitric acid.

After cooling, rinse the condensers down with 25 c.c. of water and transfer the contents of the flask to a 500 c.c. separatory funnel, with the aid of 25 c.c. more of water. The total volume of the solution is now 100 c.c. Add to the solution sufficient ammonium sulphate to just saturate it (about 55 gms.). Make four extractions with freshly washed chloroform, using 50, 35, 25, and 25 c.c. portions. The first two portions may be used to further rinse out the Kjeldahl flask.

Collect the successive portions of chloroform in another separatory funnel. Add to the combined extracts 100 c.c. of a saturated solution of pure sodium chloride, to each liter of which has been added 0.5 c.c. of concentrated HCl. Shake well, draw the chloroform into a dry 500 c.c. Erlenmeyer flask and titrate with N/10 sodium alcoholate, using 4 or 5 drops of phenolphthalein as an indicator. The first distinct end point should be taken, although it may fade on standing a short time.

Calculation.—Multiply the number of cubic centimeters of alcoholate used by the factor for hippuric acid as determined by standardization to obtain the amount of hippuric acid in the 100 c.c. of urine used. One c.c. of exactly N/10 sodium alcoholate is equivalent to 0.0179 gm. of hippuric acid. Calculate the daily output of hippuric acid from the twenty-four hour volume.

GLUCOSE

Fehling's Method

Principle.—Diluted urine is run into a measured amount of Fehling's solution at the boiling-point until all of the copper it contains is reduced as indicated by the loss of blue color. This

method has several disadvantages over Benedict's method. The end-point is difficult to determine and the mixed solution is unstable. It gives less accurate results.

Procedure.—Place 10 c.c. of the urine under examination in a 100 c.c. volumetric flask and make the volume up to 100 c.c. with distilled water. (If the urine contains less than 0.5 per cent of sugar it may be used without dilution. A concentration of about 0.5 per cent is the most satisfactory for this titration.) Thoroughly mix this diluted urine by pouring it into a beaker and stirring with a glass rod, then transfer a portion of it to a burette which is properly supported in a clamp.

Now place 10 c.c. of Fehling's solution in a small beaker, dilute it with approximately 40 c.c. of distilled water, heat to boiling, and observe whether decomposition of the Fehling's solution itself has occurred as indicated by the production of a turbidity. If such turbidity is produced the Fehling's solution is unfit for use. Clamp the burette containing the dilute urine immediately over the beaker and carefully allow from 0.5 to 1 c.c. of the diluted urine to flow into the boiling Fehling's solution. Bring the solution to the boiling-point after each addition of urine and continue running the urine from the burette, 0.5 to 1 c.c. at a time, as indicated, until the Fehling's solution is completely reduced, i.e., until all the cupric oxide in solution has been precipitated as cuprous oxide. This point will be indicated by the absolute disappearance of all blue color. When this end-point is reached note the number of cubic centimeters of diluted urine used in the process and calculate the percentage of dextrose present, in the sample of urine analyzed, according to the method given below.

This is a satisfactory method, the main objection to its use being the uncertainty attending the determination of the end-reaction, i.e., the difficulty with which the exact point where the blue color finally disappears is noted. Several means of accurately fixing this point have been suggested, but they are practically all open to objection. As good a " check " as any, perhaps, is to filter a few drops of the solution through a double paper, after the blue color has apparently disappeared, acidify the filtrate with acetic acid and add potassium ferrocyanide. If the copper of the Fehling's solution has been completely reduced, there will be no color reaction whereas the production of a brown

color indicates the presence of unreduced copper. Harrison has recently suggested the following procedure to determine the exact end-point: To about 1 c.c. of a starch iodide solution in a test-tube add 2 to 3 drops of acetic acid and introduce into the acidified mixture 1 to 2 drops of the solution to be tested. Unreduced copper will be indicated by the production of a purplish-red or blue color due to the liberation of iodine.

It is ordinarily customary to make at least three determinations by Fehling's method before coming to a final conclusion regarding the sugar content of the urine under examination.

Calculation.—Ten c.c. of Fehling's solution is completely reduced by 0.05 gm. of dextrose. If y represents the number of cubic centimeters of undiluted urine (obtained by dividing the burette reading by 10) necessary to reduce the 10 c.c. of Fehling's solution, we have the following proportion:

$$y : 0.05 : : 100 : x \text{ (percentage of dextrose).}$$

GLUCOSE

Benedict's Method [1]

Principle.—Benedict's reagent for the estimation of reducing sugars contains potassium thiocyanate as well as copper sulphate, and in the presence of the former a white precipitate of cuprous thiocyanate is formed on reduction instead of the usual red precipitate of cuprous oxide. The small amount of potassium ferrocyanide also aids in keeping cuprous oxide in solution. As the precipitate formed is white the loss of all blue tint in the solution, indicating complete reduction of the copper, is readily observed. The alkali used is sodium carbonate, which has the advantage over the hydroxide in that there is less danger of destruction of small amounts of sugar. The solution also has the great advantage of being stable for an indefinite length of time. The method is recommended for simplicity and accuracy.

Procedure.—The urine, 10 c.c. of which should be diluted with water to 100 c.c. (unless the sugar content is believed to be low, when it may be used undiluted), is poured into a 50 c.c. burette up to the zero mark. Twenty-five c.c. of the reagent

[1] Benedict: Jour. Am. Med. Ass'n., 1911, 57, 1193.

are measured with a pipette into a porcelain evaporation dish (25 to 30 cm. in diameter), 10 to 20 gms. of crystallized sodium carbonate (or one-half the weight of the anhydrous salt) are added, together with a small quantity of powdered pumice or talcum, and the mixture heated to boiling over a free flame until the carbonate has entirely dissolved. The diluted urine is now run in from the burette, rather rapidly, until a chalk-white precipitate forms and the blue color of the mixture begins to lessen perceptibly, after which the solution from the burette must be run in a few drops at a time until the disappearance of the last trace of blue color, which marks the end-point. The solution must be kept vigorously boiling throughout the entire titration. If the mixture becomes too concentrated during the process, water may be added from time to time to replace the volume lost by evaporation.

Calculation.—The calculation of the percentage of sugar in the original sample of urine is very simple. The 25 c.c. of copper solution are reduced by exactly 50 mg. of glucose. Therefore the volume run out of the burette to effect the reduction contained 50 mg. of the sugar. When the urine is diluted 1:10, as in the usual titration of diabetic urines, the formula for calculating the per cent of the sugar is the following:

$$\frac{0.050}{x} \times 1000 = \text{per cent in original sample, wherein } x$$

is the number of cubic centimeters of the diluted urine required to reduce 25 c.c. of the copper solution.

In the use of this method chloroform must not be present during the titration. If used as a preservative in the urine it may be removed by boiling a sample for a few minutes, and then diluting to its original volume.

ACETONE BODIES

Van Slyke's Methods[1]

Principle.—The method is based on a combination of Shaffer's oxidation of β-hydroxybutyric acid to acetone, and Denigè's precipitation of acetone as a basic mercuric sulphate

[1] Van Slyke: Jour. Biol. Chem., 1917, 32, 455.

compound. Glucose and certain other interfering substances are removed by precipitation with copper sulphate and calcium hydroxide. Preservatives other than toluene or copper sulphate should not be used.

Procedure.—Removal of Glucose and other Interfering Substances from Urine.

Place 25 c.c. of urine in a 250 c.c. measuring flask. Add 100 c.c. of water, 50 c.c. of copper sulphate solution and mix. Then add 50 c.c. of 10 per cent calcium hydroxide suspension, shake, and test with litmus. If not alkaline, add more calcium hydroxide. Dilute to the mark and let stand at least one-half hour for glucose to precipitate. Filter through a dry folded filter. This procedure will remove up to 8 per cent of glucose. Urine containing more should be diluted enough to bring the glucose down to 8 per cent. The copper treatment is depended upon to remove interfering substances other than glucose, and should therefore never be omitted, even when glucose is absent. The filtrate may be tested for glucose by boiling a little in a test-tube. A precipitate of yellow cuprous oxide will be obtained if the removal has not been complete. A slight precipitate of white calcium salts always forms, but does not interfere with the detection of the yellow cuprous oxide.

Determination of Total Acetone Bodies (Acetone, Acetoacetic Acid, and β-hydroxybutyric Acid.)—Place in a 500 c.c. Erlenmeyer flask 25 c.c. of urine filtrate. Add 100 c.c. of water, 10 c.c. of 50 per cent sulphuric acid, and 35 c.c. of the 10 per cent mercuric sulphate. Or in place of adding the water and reagents separately, add 145 c.c. of the "combined reagents." Connect the flask with a reflux condenser having a straight condensing tube of 8 or 10 mm. diameter and heat to boiling. After boiling has begun, add 5 c.c. of the 5 per cent dichromate through the condenser tube. Continue boiling gently $1\frac{1}{2}$ hours. The yellow precipitate which forms consists of the mercury sulphate-chromate compound of the preformed acetone, and the acetone which has been formed by decomposition of acetoacetic acid and by oxidation of the β-hydroxybutyric acid. It is collected in a Gooch or "medium density" alundum crucible, washed with 200 c.c. of cold water, and dried for an hour at 110°. The crucible is allowed to cool in room air (a desiccator is unnecessary and undesirable) and weighed. Several precipitates may be

collected, one above the other, without cleaning the crucible. As an alternative to weighing, the precipitate may be dissolved and titrated as described below.

Determination of Acetone and Acetoacetic Acid.—The acetone plus the acetoacetic acid, which completely decomposes into acetone and CO_2 on heating, is determined without the β-hydroxybutyric acid exactly as the total acetone bodies, except that (1) no dichromate is added to oxidize the β-hydroxybutyric acid and (2) the boiling must continue for not less than thirty nor more than forty-five minutes. Boiling for more than forty-five minutes splits off a little acetone from β-hydroxybutyric acid even in the absence of chromic acid.

Determination of β-hydroxybutyric Acid.—The β-hydroxybutyric acid alone is determined exactly as total acetone bodies except that the preformed acetone and that from the acetoacetic acid are first boiled off. To do this the 25 c.c. of urine filtrate plus 100 c.c. of water are treated with 2 c.c. of the 50 per cent sulphuric acid and boiled in the open flask for ten minutes. The volume of solution left in the flask is measured in a cylinder. The solution is returned to the flask, and the cylinder washed with enough water to replace that boiled off and restore the volume of the solution to 127 c.c. Then 8 c.c. of the 50 per cent sulphuric acid and 35 c.c. of mercuric sulphate are added. The flask is connected under the condenser and the determination is continued as described for total acetone bodies.

Titration of the Precipitate in the above Methods.—Instead of weighing the precipitate, one may wash the contents of the Gooch, including the asbestos, into a small beaker with as little water as possible, and add 15 c.c. of normal HCl. The mixture is then heated, and the precipitate quickly dissolves. In case an alundum crucible is used, it is set into the beaker of acid until the precipitate dissolves, and then washed with suction, the washings being added to the beaker. In place of using either a Gooch or alundum crucible one may, when titration is employed, wash the precipitate without suction on a small quantitative filter paper, which is transferred with the precipitate to the beaker and broken up with a rod in 15 c.c. of normal HCl.

In order to obtain a good end-point in the subsequent titration it is necessary to reduce the acidity of the solution. For this purpose it has been found that the addition of excess sodium

acetate is the most satisfactory means. Six to 7 c.c. of 3 M acetate are added to the cooled solution of redissolved precipitate. Then the 0.2 M KI is run in rapidly from a burette with constant stirring. If more than a small amount of mercury is present, a red precipitate of HgI_2 at once forms, and redissolves as soon as 2 or 3 c.c. of KI in excess of the amount required to form the soluble K_2HgI_4 have been added. If only a few mg. of mercury are present, the excess of KI may be added before the HgI_2 has had time to precipitate so that the titrated solution remains clear. In this case not less than 5 c.c. of the 0.2 M KI are added, as it has been found that the final titration is not satisfactory if less is present. The excess of KI is titrated back by adding 0.05 M $HgCl_2$ from another burette until a permanent red precipitate forms. Since the reaction utilized is $HgCl_2+4KI=K_2HgI_4+KCl$, 1 c.c. of 0.05 M $HgCl_2$ is equivalent in the titration to 1 c.c. of the 0.2 M KI.

In preparing the two standard solutions the 0.05 M $HgCl_2$ is standardized by the sulphide method, and the iodine is standardized by titration against it. A slight error appears to be introduced if the iodide solution is gravimetrically standardized and used for checking the mercury solution, instead of vice versa.

In standardizing the mercuric chloride the following procedure has been found convenient: 25 c.c. of 0.05 M $HgCl_2$ are measured with a calibrated pipette, diluted to about 100 c.c., and H_2S is run in until the black precipitate flocculates and leaves a clear solution. The HgS, collected in a Gooch crucible and dried at 110°, should weigh 0.2908 gm. if the solution is accurate.

Both by gravimetric analyses of the basic mercuric sulphate-acetone precipitate and by titration, the mercury content of the precipitate has been found to average 76.9 per cent. On this basis, each c.c. of 0.2 M KI solution, being equivalent to 10.0 mg. of Hg, is equivalent to 13.0 mg. of the mercury acetone precipitate.

Titration is not quite so accurate as weighing, but, except when the amounts determined are very small, the titration is satisfactory.

Calculation.—1 mg. of β-hydroxybutyric acid yields 8.45 mg. of precipitate. 1 mg. of acetone yields 20.0 mg. of precipitate. 1 c.c. of 0.2 M KI solution is equivalent to 13 mg. of precipitate in titration of the latter.

SPECIAL FACTORS FOR CALCULATION OF RESULTS WHEN 25 c.c. OF URINE FILTRATE, EQUIVALENT TO 2.5 c.c. OF URINE, ARE USED FOR THE DETERMINATION

Determination Performed	Acetone Bodies, Calculated as Gm. Acetone per Liter of Urine, Indicated by:	
	1 gm. of prec.	1 c.c. of 0.2 M KI sol.
Total acetone bodies	24.8	0.322
β-Hydroxybutyric acid	26.4	0.344
Acetone, acetoacetic acid	20.0	0.260

In order to calculate the acetone bodies as β-hydroxybutyric acid rather than acetone, use the above factors multiplied by the ratio of the molecular weights $\frac{\beta\text{-acid}}{\text{acetone}} = \frac{104}{58} = 1.793$. In order to calculate the acetone bodies in terms of molecular concentration, divide the factors in the table by 58. To calculate c.c. of 0.1 M acetone bodies per liter of urine use the above factors multiplied by $\frac{10{,}000}{58} = 172.4$.

ACETONE

Folin's Method[1]

Principle.—The preformed acetone is aspirated from the urine mixture at room temperature to prevent decomposition of acetoacetic acid. The acetone is collected in alkaline hypoiodite solution as in the Folin-Hart method. Iodoform is formed quantitatively and the excess of iodine is titrated with sodium thiosulphate.

Procedure.—The same type of apparatus is used in this method as that described in Folin's method for the determination of ammonia (see page 42). Introduce 20 to 25 c.c. of the urine under examination into the aerometer cylinder and add 10 drops

[1] Folin: Jour. Biol. Chem., 1914, 18, 263.

of 10 per cent phosphoric acid, 8 to 10 gms. of sodium chloride, and a little petroleum. Introduce into an absorption flask, such as is used in the ammonia determination (see page 42), 150 c.c. of water, 10 c.c. of a 40 per cent solution of potassium hydroxide, and an excess of a N/10 iodine solution. Connect the flask with the aerometer cylinder, attach a Chapman pump, and permit an air current, slightly less rapid than that used for the determination of ammonia, to be drawn through the solution for twenty to twenty-five minutes. All of the acetone will, at this point, have been converted into iodoform in the absorption flask. Add 10 c.c. of concentrated hydrochloric acid (a volume equivalent to that of the strong alkali originally added), to the contents of the latter and titrate the excess of iodine by means of N/10 sodium thiosulphate solution until a light yellow color is obtained. At this point add a few cubic centimeters of starch paste and titrate until no blue color is visible. This is the end reaction.

Calculation.—Subtract the number of cubic centimeters of N/10 thiosulphate solution used from the volume of N/10 iodine solution employed. Since 1 c.c. of the iodine solution is equivalent to 0.967 mg. of acetone, and since 1 c.c. of the thiosulphate solution is equivalent to 1 c.c. of the iodine solution, if we multiply the remainder from the above subtraction by 0.967 we will obtain the number of milligrams of acetone in the volume of urine employed.

Calculate the quantity of acetone in the twenty-four hour urine specimen.

Folin has further made suggestions regarding the *simultaneous* determination of acetone and ammonia by the use of the same air current. This is an important consideration for the clinician inasmuch as urines which contain acetone and acetoacetic acid are generally those from which the ammonia data are also desired. The procedure for the combination method is as follows: Arrange the ammonia apparatus as usual (see page 42), and to the aerometer of the ammonia apparatus attach the acetone apparatus set up as described above. Regulate the air current with special reference to the determination of acetone and at the end of twenty to twenty-five minutes disconnect the acetone apparatus and complete the determination of the acetone as just described. The air current is not interrupted, and after having run one and one-half

hours the ammonia apparatus is detached and the ammonia determination completed as described on page 42.

If data regarding acetoacetic acid are desired, the result obtained by Folin's method may be subtracted from the result obtained by the Van Slyke method for acetone and acetoacetic acid. Under all conditions the determination of acetone should be as expeditious as possible. This is essential, not only because of the fact that any acetoacetic acid present in the urine will become transformed into acetone, but *also* because of the rapid spontaneous decomposition of the alkaline hypoiodite solution used in the determination of the acetone. It has been claimed that alkaline hypoiodite solutions are almost completely converted into *iodate* solutions in *one-half hour*. Folin states, however, that the transformation is not so rapid as this, but he nevertheless emphasizes the necessity of rapidity of manipulation. At the same time it should be remembered that the air current must not be as rapid as for ammonia, inasmuch as the alkaline hypoiodite solution will not absorb all the acetone under those conditions.

INDICAN

Ellinger's Method [1]

Principle.—This method for the quantitative determination of indican is based upon the principle underlying Jaffé's qualitative test for indican. The urine after removal of interfering substances with basic lead acetate is treated with Obermayer's reagent to oxidize the indican to indigo. The indigo is extracted with chloroform, the chloroform evaporated off and the residue titrated with potassium permanganate. The method is not very accurate but is as satisfactory as any.

Procedure.—To 50 c.c. of urine in a small beaker or casserole add 5 c.c. of basic lead acetate solution, mix thoroughly, and filter. Transfer 40 c.c. of the filtrate to a separatory funnel, add an equal volume of Obermayer's reagent and 20 c.c. of chloroform, and extract in the usual manner. This extraction with chloroform should be repeated until the chloroform solution remains colorless. Shake up the combined chloroform extracts two or three times with distilled water in a separating funnel and complete the

[1] Hawk: Practical Physiological Chemistry, Sixth Edition, 1918.

purification by extracting with very dilute sodium hydroxide (1:1000). Remove all traces of alkali by washing with water. Now filter the combined chloroform extracts through a dry filter paper into a dry Erlenmeyer flask. Distill off the chloroform, heat the residue on a boiling water-bath for five minutes in the open flask, and wash the dried residue with hot water. Add 10 c.c. of concentrated sulphuric acid to the washed residue, heat on the water-bath for five to ten minutes, dilute with 100 c.c. of water, and titrate the blue solution with a very dilute solution of potassium permanganate. The end-point is indicated by the dissipation of all the blue color from the solution and the formation of a pale yellow color.

Beautiful plates of indigo blue sometimes appear in the chloroform extract of urines containing abundant indican. In urines preserved by thymol the determination of indican is interfered with unless great care is taken in washing the chloroform extract with dilute alkali. Care should be taken, therefore, to make the indican determination upon fresh urine, before the addition of the preservative.

Plasencia has suggested a method which is shorter than Ellinger's and according to its sponsor, just as accurate.

Calculation.—One cubic centimeter of the diluted permanganate solution is equivalent to about 0.15 mg. of indigo. Ellinger claims that one-sixth of the amount determined must be added to the value obtained by titration in order to secure accurate data. This correction should always be made.

PHENOLS

Colorimetric Method of Folin and Denis[1]

Principle.—This method is based upon the fact that phenols yield with a solution of phosphotungstic-phosphomolybdic acid and alkali a deep blue color the depth of which is proportional to the amount of such substances present. Traces of protein, which may be present in the urine, and uric acid give a blue color with the reagent and are removed by precipitation with an ammoniacal silver solution and colloidal iron as a preliminary to the determination of the phenols.

[1] Folin and Denis: Jour. Biol. Chem., 1915, 22, 305.

Procedure.—*Removal of Interfering Substances.*—Place 10 c.c. of ordinary urine, or 20 c.c. of a dilute urine in a 50 c.c. volumetric flask. To this add an acid silver lactate solution (from 2 to 20 c.c. of a 3 per cent solution of silver lactate in 3 per cent lactic acid) until no further precipitate is obtained. Add a few drops of colloidal iron, shake the flask, dilute to mark with distilled water, shake again, and filter the contents through a dry filter. Phenols are not precipitated by this procedure but are recovered quantitatively in the filtrate. Transfer 25 c.c. of the filtrate to a 50 c.c. volumetric flask, and add a sufficient quantity of saturated sodium chloride solution, containing 10 c.c. of strong hydrochloric acid per liter, to precipitate all the silver. Fill the flask to the mark with distilled water, mix thoroughly, and filter through a dry filter. This filtrate, which contains half the phenol from the urine taken for analysis, is used for the determination of free and total phenols.

Free Phenols.—Place 20 c.c. of the filtrate mentioned above in a 50 c.c. volumetric flask, add 5 c.c. of the phosphotungstic-phosphomolybdic acid reagent and 15 c.c. of a saturated solution of sodium carbonate. Dilute to volume with lukewarm water (30 to 35° C.), mix thoroughly and after allowing to stand for twenty minutes compare the deep blue color in the Duboscq colorimeter against a standard solution of phenol (see below) similarly treated.

Total Phenols (Free and Conjugated).—Place 20 c.c. of the same filtrate used for the determination of free phenols in a large test-tube, add 10 drops of concentrated hydrochloric acid, cover the tube with a small funnel, heat rapidly to boiling over a free flame, and then place in a boiling water-bath for ten minutes. This process serves to decompose the conjugated phenols. At the end of the ten minutes, remove the tube, cool, and transfer the contents to a 100 c.c. volumetric flask. Add 10 c.c. of the phosphotungstic-phosphomolybdic reagent, 25 c.c. of saturated sodium carbonate solution, dilute to mark with lukewarm water (30 to 35° C.), mix thoroughly, allow to stand for twenty minutes, and read in the Duboscq colorimeter against a standard solution of phenol (see below).

Standard Solution of Phenol.—The standard used is a solution of pure phenol in N/100 hydrochloric acid containing 1 mg. of phenol in 10 c.c. standardized by means of the iodometric titration.

The preparation is carried out as follows: Make a phenol solution in N/10 hydrochloric acid, which contains approximately 1 mg. of crystallized phenol per cubic centimeter. Transfer 25 c.c. of this solution to a 250 c.c. flask, add 50 c.c. of N/10 sodium hydroxide, heat to 65° C., add 25 c.c. of N/10 iodine solution, stopper the flask, and let stand at room temperature thirty or forty minutes. Add 5 c.c. of concentrated hydrochloric acid and titrate the excess of iodine with N/10 thiosulphate solution. Each cubic centimeter of N/10 iodine solution corresponds to 1.567 mg. of phenol. On the basis of the result dilute the phenol solution so that 10 c.c. contain 1 mg. of phenol. Five c.c. of this solution (equivalent to 0.5 mg. of phenol), when 10 c.c. of the phosphotungstic-phosphomolybdic reagent and 25 c.c. of saturated sodium carbonate solution are added, and the whole made up with water at about 30° C. to 100 c.c. give when set in the colorimeter at 20 mm. a convenient standard.

Calculation.—The filtrate used for the determination of free and total phenols contains the phenols from one-half the amount of urine analyzed. The actual determination of phenols, both free and total, is made upon a two-fifths portion of this filtrate, and this amount of filtrate contains the phenols from one-fifth of the amount of urine analyzed. In the determination of free phenols the colored solution is diluted to only half that of the standard while in the determination of total phenols the dilution is the same as that of the standard.

Hence,

$$\frac{R_1}{R_2 \times 4} = \text{milligrams of free phenol}$$

and

$$\frac{R_1}{R_2 \times 2} = \text{milligrams of total phenol}$$

in 2 c.c. or 4 c.c. of urine according to whether 10 c.c. or 20 c.c. of urine was taken for analysis, when R_1 is taken as the reading obtained with the standard solution, and R_2 is taken as the reading obtained with the unknown.

TOTAL SULPHUR

Benedict's Method [1]

Principle.—The urine is evaporated and ignited with a solution of copper nitrate and potassium chlorate. Organic matter is thus destroyed and all unoxidized sulphur is oxidized to the sulphate form and can be readily precipitated with barium chloride in the usual manner. The method is very convenient and accurate.

Procedure.—Ten c.c. of urine are measured into a small (7–8 cm.) porcelain evaporating dish and 5 c.c. of Benedict's sulphur reagent added. The contents of the dish are evaporated over a free flame which is regulated to keep the solution just below the boiling-point, so that there can be no loss through spattering. When dryness is reached, the flame is raised slightly until the entire residue has blackened. The flame is then turned up in two stages to the full heat of the Bunsen burner and the contents of the dish thus heated to redness for ten minutes after the black residue (which first fuses) has become dry. This heating is to decompose the last traces of nitrate (and chlorate). The flame is then removed and the dish allowed to cool more or less completely. Ten to 20 c.c. of dilute (1:4) hydrochloric acid is then added to the residue in the dish, which is then warmed gently until the contents have completely dissolved and a perfectly clear, sparkling solution is obtained. This dissolving of the residue requires scarcely two minutes. With the aid of a stirring rod the solution is washed into a small Erlenmeyer flask, diluted with cold, distilled water to 100 to 150 c.c., 10 c.c. of 10 per cent barium chloride solution added drop by drop, and the solution allowed to stand for about an hour. It is then shaken up and filtered as usual through a weighed Gooch crucible. Controls should be run on the oxidizing mixture.

Calculation.—Make the calculation according to directions given under Total Sulphates, page 73. Calculate the quantity of sulphur expressed as SO_3 or S, present in the twenty-four-hour urine specimen.

[1] Benedict: Jour. Biol. Chem., 1909, 6, 363.

TOTAL SULPHATES

Folin's Method[1]

Principle.—The sulphuric acid of the conjugated sulphates is set free by boiling with acid. The total sulphates are then precipitated with barium chloride.

Procedure.—Place 25 c.c. of urine in a 200 to 250 c.c. Erlenmeyer flask, add 20 c.c. of dilute hydrochloric acid (1 volume of concentrated HCl to 4 volumes of water) and gently boil the mixture for twenty to thirty minutes. To minimize the loss of water by evaporation the mouth of the flask should be covered with a small watch-glass during the boiling process. Cool the flask for two to three minutes in running water, and dilute the contents to about 150 c.c. by means of cold water. Add 10 c.c. of a 5 per cent solution of barium chloride slowly, drop by drop, to the cold solution. The contents of the flask should not be stirred or shaken during the addition of the barium chloride. Allow the mixture to stand at least one hour, then shake up the solution and filter it through a weighed Gooch crucible.

Wash the precipitate of $BaSO_4$ with about 250 c.c. of cold water, dry it in an air-bath or over a very low flame, then ignite, cool, and weigh.

Calculation.—Subtract the weight of the Gooch crucible from the weight of the crucible and the $BaSO_4$ precipitate to obtain the weight of the precipitate. The weight of SO_3 or S in the volume of urine taken may be determined by means of the following proportion:

Mol. Wt. $BaSO_4$: Wt. of $BaSO_4$ :: Mol. Wt. SO_3 : $\times$ (wt. of SO_3 in grams).

Representing the weight of the $BaSO_4$ precipitate by y and substituting the proper molecular weights, we have the following proportion:

$233.43 : y :: 80.06 : x$ (wt. of SO_3 in grams in the quantity of urine used).

Calculate the quantity of SO_3 in the twenty-four-hour specimen of urine.

[1] Folin: Jour. Biol. Chem., 1905, 1, 131.

To express the result in percentage of SO_3 simply divide the value of x, as just determined, by the quantity of urine used.

INORGANIC SULPHATES[1]

Folin's Method

Procedure.—Place 25 c.c. of urine and 100 c.c. of water in a 200 to 250 c.c. Erlenmeyer flask and acidify the diluted urine with 10 c.c. of dilute hydrochloric acid (1 volume of concentrated HCl to 4 volumes of water). In case the urine is dilute 50 c.c. may be used instead of 25 c.c. and the volume of water reduced proportionately. Add 10 c.c. of 5 per cent barium chloride slowly, drop by drop, to the cold solution and from this point proceed as indicated in the method for the determination of Total Sulphates, page 73.

Calculate the quantity of inorganic sulphates, expressed as SO_3, in the twenty-four-hour urine specimen.

Calculation.—Calculate according to the directions given under Total Sulphates, page 73.

ETHEREAL SULPHATES[2]

Folin's Method

Principle.—The inorganic sulphates are removed with barium chloride and the conjugated sulphates then determined after hydrolysis.

Procedure.—Place 125 c.c. of urine in an Erlenmeyer flask of suitable size, dilute it with 75 c.c. of water and acidify the mixture with 30 c.c. of dilute hydrochloric acid (1 volume of concentrated HCl to 4 volumes of water). To the cold solution add 20 c.c. of a 5 per cent solution of barium chloride, drop by drop. Allow the mixture to stand about one hour, then filter it through a dry filter paper. Collect 125 c.c. of the filtrate and boil it gently for at least one-half hour. Cool the solution, filter off the precipitate of $BaSO_4$, wash, dry and ignite it according to the directions given on page 73.

[1] Folin: Jour. Biol. Chem., 1905, 1, 131.
[2] Ibid.

Calculation.—The weight of the $BaSO_4$ precipitate should be multiplied by 2, since only one-half (125 c.c.) of the total volume (250 c.c.) of fluid was precipitated by the barium chloride. The remaining calculation should be made according to directions given under Total Sulphates, page 73.

Calculate the quantity of ethereal sulphates, expressed as SO_3, in the twenty-four-hour urine specimen.

PHOSPHORUS

Total Phosphates (Uranium Acetate Method)

Principle.—Standard uranium acetate is run into a measured quantity of urine until all of the phosphate has been precipitated as insoluble uranium phosphate. An excess of uranium is indicated by a reddish coloration with potassium ferrocyanide. This method is accurate and gives practically the total phosphorus of urine inasmuch as the latter exists generally almost entirely as phosphates.

Procedure.—To 50 c.c. of urine in a small beaker or Erlenmeyer flask add 5 c.c. of a special sodium acetate solution and heat the mixture to the boiling-point. From a burette, run into the hot mixture, drop by drop, a standard solution of uranium acetate until a precipitate ceases to form and a drop of the mixture when removed by means of a glass rod and brought into contact with a drop of a solution of potassium ferrocyanide on a porcelain test-plate produces instantaneously a brownish-red coloration. Take the burette reading and calculate the P_2O_5 content of the urine under examination.

Calculation.—Multiply the number of cubic centimeters of uranium acetate solution used by 0.005 to determine the number of grams of P_2O_5 in the 50 c.c. of urine used. To express the result in percentage of P_2O_5 multiply the value just obtained by 2, e.g., if 50 c.c. of urine contained 0.074 gm. of P_2O_5 it would be equivalent to 0.148 per cent.

Calculate, in terms of P_2O_5, the total phosphate content of the twenty-four-hour urine specimen.

CHLORIDES

Volhard-Arnold Method [1]

Principle.—The urine is acidified with nitric acid and the chlorides precipitated with a measured excess of standard silver nitrate solution. The silver chloride formed is filtered off and in the filtrate the excess silver nitrate is titrated back with standard ammonium thiocyanate solution. Ferric ammonium sulphate is used as an indicator. A red color due to the formation of ferric thiocyanate indicates that an excess of thiocyanate is present and that the end-point has been reached.

Procedure.—Place 10 c.c. of urine in a 100 c.c. volumetric flask, add 20 to 30 drops of nitric acid (sp. gr. 1.2) and 2 c.c. of a cold saturated solution of ferric alum. If necessary, at this point a few drops of 8 per cent solution of potassium permanganate may be added to dissipate the red color. Now slowly run in a known volume of the standard silver nitrate solution (20 c.c. is ordinarily used) in order to precipitate the chlorine and insure the presence of an excess of silver nitrate. The mixture should be continually shaken during the addition of the standard solution. Allow the flask to stand ten minutes, then fill it to the 100 c.c. graduation with distilled water and thoroughly mix the contents. Now filter the mixture through a dry filter paper, collect 50 c.c. of the filtrate and titrate it with standardized ammonium thiocyanate solution. The first permanent tinge of red-brown indicates the end-point. Take the burette reading and compute the weight of sodium chloride in the 10 c.c. of urine used.

Calculation.—The number of cubic centimeters of ammonium thiocyanate solution used indicates the excess of standard silver nitrate solution in the 50 c.c. of filtrate titrated. Multiply this reading by 2, inasmuch as only one-half of the filtrate was employed, and subtract this product from the number of cubic centimeters of silver nitrate (20 c.c.) originally used, in order to obtain the actual number of cubic centimeters of silver nitrate utilized in the precipitation of the chlorides in the 10 c.c. of urine employed.

To obtain the weight in grams of the sodium chloride in the 10 c.c. of urine used, multiply the number of cubic centimeters of

[1] Hawk: Practical Physiological Chemistry, Sixth Edition, 1918.

the standard silver nitrate solution, actually utilized in the precipitation, by 0.010. If it is desired to express the result in percentage of sodium chloride move the decimal point one place to the right.

In a similar manner the weight, or percentage of chlorine may be computed using the factor 0.006 instead of 0.010.

Calculate the quantity of sodium chloride and chlorine in the twenty-four-hour urine specimen.

CHLORIDES

Volhard-Harvey Method [1]

Principle.—This procedure differs from the Volhard-Arnold method in that the excess of silver nitrate is titrated directly without filtering and hence in the presence of the silver chloride. The procedure is thus more rapid, but the exact end-point is more difficult to determine.

Procedure.—Introduce 5 c.c. of urine into a small porcelain evaporating dish or casserole and dilute with about 20 c.c. of distilled water. Precipitate the chlorides by the addition of 10 c.c. of standard silver nitrate solution and add 2 c.c. of acidified indicator. Now run in a standard ammonium thiocyanate solution from a burette until a faint red-brown tint is visible throughout the mixture. This point may be determined readily by permitting the precipitate to settle somewhat. Calculate the sodium chloride value as indicated below.

(If a red tint is produced when the first drop of thiocyanate is added an additional 10 c.c. of the standard silver nitrate solution must be introduced. The titration should then proceed as above described and proper allowance made in the calculation for the extra volume of silver nitrate employed.)

Calculation.—Since 2 c.c. of the ammonium thiocyanate solution is equivalent to 1 c.c. of the silver nitrate solution, divide the burette reading by 2 and subtract the quotient from 10 c.c., the quantity of silver nitrate solution taken. This value is the number of cubic centimeters of silver nitrate solution actually used in the precipitation of the chlorides. As 1 c.c. of the silver nitrate solution is equivalent to 0.01 gm. of sodium chloride, the number of

[1] Harvey: Arch. Int. Med., 1910, 6, 12.

cubic centimeters of silver nitrate solution used multiplied by 0.01 gm. will give the weight of sodium chloride in the 5 c.c. portion of urine used. The weight of chlorine may be computed by using the factor 0.006 instead of 0.01.

Calculate the weight of sodium chloride and chlorine in the twenty-four-hour urine specimen.

A "short cut" method of calculating the twenty-four-hour output of sodium chloride consists in subtracting the burette reading from 20 c.c., multiplying this value by the total urine volume and pointing off three places.

CALCIUM AND MAGNESIUM

McCrudden's Methods [1]

Principle.—Urine contains magnesium, phosphates, and a small amount of iron, each of which will interfere with the accurate determination of its calcium content if proper conditions of acidity are not maintained during the precipitation. In the following method the proper acidity is attained through the use of sodium acetate and hydrochloric acid, and this with slow addition of the ammonium oxalate reduces the danger of occlusion of magnesium oxalate, calcium phosphate, or ferric phosphate in the calcium oxalate precipitate.

The calcium oxalate precipitate is either ignited and weighed as CaO or determined volumetrically by titration with potassium permanganate. Magnesium is determined in the filtrate from the calcium determination after destruction of the organic matter. It is determined in the usual way by ignition of the magnesium ammonium phosphate precipitate and weighing as the pyrophosphate.

Lyman has suggested a nephelometric method for the determination of calcium in urine and feces.

Procedure for Calcium.—If the urine is alkaline, make it neutral or slightly acid and filter. Take 200 c.c. of the filtered urine for analysis. If it is only faintly acid to litmus paper, add 10 drops of concentrated hydrochloric acid (sp.gr. 1.20). If the urine is strongly acid, it may be made just alkaline with ammonia and then just acid with hydrochloric acid after which the 10 drops of

[1] McCrudden: Jour. Biol. Chem., 1910, 7, 83; 1911, 10, 187.

concentrated hydrochloric acid are added. Then add 10 c.c. of 2.5 per cent oxalic acid. Run in slowly with stirring 8 c.c. of 20 per cent sodium acetate. Allow to stand over night at room temperature or shake vigorously for ten minutes. Filter off the precipitate of calcium oxalate on a small paper and wash free from chlorides with 0.5 per cent ammonium oxalate solution. The precipitate may then be dried, ignited to constant weight and weighed as calcium oxide or it may be manipulated volumetrically as described below.

Volumetric Procedure.—If free from uric acid, the calcium oxalate precipitate may be washed three times with distilled water, filling the filter about two-thirds full and allowing it to drain completely before adding more. A hole is made in the paper and the calcium oxalate washed into the flask. The volume of the fluid is brought up to about 50 c.c. and 10 c.c. of concentrated sulphuric acid added. Titrate with standard potassium permanganate solution to a pink color which endures for at least a minute.

Calculation.—One c.c. of N/10 permanganate solution is equivalent to 2.8 mg. of CaO. Calculate the daily output of calcium expressed as CaO.

Procedure for Magnesium.—Transfer the filtrate from the determination of calcium as above to a porcelain dish, add about 20 c.c. of concentrated nitric acid and evaporate to dryness. Heat the residue over a free flame until the ammonium salts are destroyed and the residue fuses. After cooling take the residue up with water and a little hydrochloric acid and filter if necessary. Dilute to about 80 c.c., nearly neutralize with ammonia and cool. Add a slight excess of sodium acid phosphate, and then ammonia drop by drop with constant stirring, until the solution is alkaline, and then add enough more slowly with constant stirring to make the solution contain one-fourth its bulk of dilute ammonia (sp.gr. 0.96). Allow to stand over night. Filter and wash free from chlorides with alcoholic ammonia solution (1 part alcohol, 1 part dilute ammonia, 3 parts water). The precipitate, with filter paper, is incinerated slowly and carefully with good supply of air to prevent reduction, in the usual manner, and ignited and weighed as the pyrophosphate.

Calculation.—To obtain the weight of MgO multiply the weight of magnesium pyrophosphate by 0.3624.

Determination of Calcium in Ash of Foods or Feces [1]

Ignite the material in a crucible to a white ash and dissolve the ash with the aid of a little hydrochloric acid. Bring the volume of the ash solution to 75 to 150 c.c. Make just alkaline with strong ammonia added drop by drop (using litmus paper or alizarin as an indicator). Add concentrated HCl drop by drop until just acid to litmus. Then add 10 drops of concentrated HCl (sp.gr. 1.20), and 10 c.c. of 2.5 per cent oxalic acid. Either of two procedures may then be followed. (*a*) The solution is boiled until the precipitated calcium oxalate is coarsely crystalline, and then an excess of 3 per cent ammonium oxalate is slowly added to the boiling solution and the boiling continued until the precipitate is coarsely crystalline. (If but little calcium is present, nothing will precipitate at this point and it is not necessary to add oxalate.) Or (*b*) the flask closed with a rubber stopper is shaken vigorously for ten minutes. An excess of 3 per cent ammonium oxalate is then added. Cool to room temperature. Add 8 c.c. of 20 per cent sodium acetate solution. (In case of ash of feces add 15 c.c.) The solution may either be (*a*) allowed to stand over night or (*b*) stoppered and vigorously shaken for ten minutes. The calcium oxalate is filtered off on a small ash free paper and washed free from chlorides with 0.5 per cent ammonium oxalate solution. Either of two procedures may next be followed. (*a*) The precipitate and filter are dried, burned in a platinum or porcelain crucible to constant weight as CaO. (*b*) The precipitate is washed three times with cold distilled water, as given under the method for urine, and the oxalate titrated with potassium permanganate.

Magnesium is determined in the filtrate from calcium just as given above.

IRON

Method of Wolter [2]

Principle.—The urine is ashed, the ash dissolved, and the iron present oxidized to the ferric iron form by means of hydrogen peroxide. The iron is then determined iodometrically.

Procedure.—The twenty-four-hour specimen of urine is treated with 30 c.c. of concentrated iron-free nitric acid and then evapo-

[1] McCrudden: Jour. Biol. Chem., 1910, 7, 83; 1911, 10, 187.

[2] Wolter: Biochem. Zeit., 1910, 24, 103.

rated to low volume in a large evaporating dish on the water-bath. Transfer to a small evaporating dish. Heat to dryness on the sand-bath and then char, using a small flame. Transfer the charred mass by means of a glass spatula to a crucible. The remaining material in the evaporating dish is transferred with the aid of a little hot water and a rubber "policeman" to a second crucible. Evaporate to dryness on the water-bath and then ash the material in both crucibles. Dissolve the ash in about 30 c.c. of iron-free hydrochloric acid, transfer to an Erlenmeyer flask, add 2 c.c. of hydrogen peroxide and boil for three-quarters of an hour. After cooling, 2 gms. of potassium iodide and a few drops of fresh starch paste are added. The liberated iodine is titrated with N/100 thiosulphate solution. Controls should be run on reagents. A correction of 0.32 mg. is usually necessary for the undecomposed hydrogen peroxide. The thiosulphate solution is made up as needed from an N/10 stock solution by dilution. It is standardized against an iron solution containing 2 mg. of iron in 10 c.c. The number of cubic centimeters of thiosulphate used in titration of the iodine set free from the ash solution is multiplied by the iron equivalent of 1 c.c. of the thiosulphate (about 0.2 mg.) to obtain the total amount of iron in the twenty-four-hour specimen of urine. From 1-5 mg. of iron are usually excreted per day.

PHENOLSULPHONEPHTHALEIN TEST FOR KIDNEY EFFICIENCY [1]

This test for renal function was devised by Rowntree and and Geraghty.[2] It depends upon the injection into the tissues of a dyestuff which is eliminated rapidly by the normal kidneys, and can be easily estimated quantitatively in the urine.

This dyestuff, phenolsulphonephthalein, is non-irritative to the body either when taken by mouth or when injected into the tissues, so that it does no harm to an already weakened kidney.

The patient upon whom the test is to be performed is given 300-400 c.c. of water twenty to thirty minutes previously, in order to assure a free flow of urine.

The procedure is as follows: One c.c. of a solution containing

[1] Hawk: Practical Physiological Chemistry, Sixth Edition, 1918.

[2] Rowntree and Geraghty: Jour. Pharm. and Exper. Therap., 1910, 1, 579: also Arch. Int. Med., March, 1912, p. 284.

6 mg. of phenolsulphonephthalein [1] is injected intramuscularly in the lumbar region, the time of injection being noted. The patient is then catheterized and the urine as it forms thereafter allowed to drop into a beaker containing 2 drops of 25 per cent NaOH. The appearance of a red color in the alkalinized urine indicates beginning excretion of the drug, the normal time being within five to ten minutes after its injection. Urine is now collected in one-hour samples. In patients with obstruction to the flow of urine from the bladder the retention catheter is stoppered and the urine drawn off at the end of each hour. Other patients may simply be allowed to urinate at the hourly periods.

To each hour sample of urine is added 25 per cent NaOH, drop by drop, until the maximum intensity of color appears. This color will remain constant for an indefinite period of time. Each sample is then placed in a 1000 c.c. volumetric flask and diluted to the mark with distilled water.

Comparison is made in a Duboscq or Hellige colorimeter with a standard consisting of 3 mg. of phenolsulphonephthalein in 1000 c.c. of solution. The cylinder containing the standard may conveniently be placed at the 10 mm. mark. Since the volume of each urine sample is the same as that of the standard, the percentage elimination of phenolsulphonephthalein in each may be easily calculated as follows:

$$\text{Reading of Urine} : \text{Reading of Standard} :: 100 : x.$$

The amount of the drug eliminated normally is 40 to 60 per cent during the first hour and 20 to 25 per cent during the second hour, or a total of 60 to 85 per cent for two hours. The amount of the drug excreted has been found to be independent of the quantity of urine obtained. In case of delayed excretion the collection of hourly samples may be continued until practically all of the drug has been recovered in the urine.

If it is desired to test the function of each kidney separately, ureteral catheterization must be resorted to, the experiment otherwise being performed as above described.

[1] This solution is prepared by adding 0.6 gm. phenolsulphonephthalein and 0.84 c.c. of 2/N NaOH to enough 0.75 per cent NaCl solution to make 100 c.c. This gives the mono-sodium or acid salt which is slightly irritant locally when injected. It is necessary to add 2 to 3 drops more 2/N NaOH which changes the color to a Bordeaux red. This preparation is non-irritant.

PART III

METHODS FOR THE ANALYSIS OF BLOOD

TOTAL SOLIDS

If total solid determination only is desired, approximately 0.5 to 1.0 gm. of blood is drawn into weighed aluminum dishes with tightly fitting covers. After a second weighing the covers are removed and the blood is dried to constant weight in an oven at a temperature of 105° to 110° C. Usually drying for a period of three hours is sufficient. Place in desiccator for one-half hour. Weigh. If it is desired to make a determination of the ash on the same sample the blood should be drawn in small porcelain crucibles.

ASH DETERMINATION

The dried, weighed blood is heated in a weighed porcelain crucible over a low flame until the ash is white on cooling. The temperature should be such that the bottom of the crucible shows only a dull red glow. Heating too rapidly will cause a loss of ash owing to volatilization of the chlorides. After the ashing is complete place crucible in desiccator for one-half hour. Weigh.

BLOOD ANALYSIS

System of Folin and Wu [1]

For the estimation of Non-Protein Nitrogen, Urea, Creatinine, Creatine, Uric Acid and Sugar.

In this system of blood analysis for the above subjects a sufficiently large quantity of blood, namely, 10 c.c. or more, is

[1] Folin and Wu: Jour. Biol. Chem., 1919, 38, 81.

freed from protein and the protein-free filtrate is used for the determination of the constituents designated above.

Method of Drawing Blood.—Attach, by means of a short piece of pure gum tubing, an hypodermic needle about 1 mm. in diameter and 25 mm. in length, previously sterilized and paraffined, to the tip of a suitable graduated pipette. Introduce into the upper end of the pipette, which must be perfectly clean and dry, a small pinch of powdered potassium oxalate, and allow it to run down into the tip and the needle. An excess of oxalate is to be avoided. Citrate should be employed only in minimum amounts. Attach a piece of rubber tubing to the upper end of the pipette, and to this a mouthpiece consisting of a short tapering glass tube. Place a pinchcock over the rubber tube near the top of the pipette. To draw the blood, insert the needle into the vein or artery and regulate the flow by means of the pinchcock and suction. The exact quantity of blood desired may thus be obtained without any waste or clotting.

Preparation of Protein-free Blood Filtrates.—Transfer a measured amount of blood into a flask having a capacity of 15 to 20 times that of the volume taken. Dilute the blood with 7 volumes of water and mix. With an appropriate pipette add 1 volume of 10 per cent solution of sodium tungstate ($Na_2WO_4.2H_2O$) and mix. With another suitable pipette add to the contents in the flask (with shaking) 1 volume of 2/3 normal sulphuric acid. Close the mouth of the flask with a rubber stopper and give a few vigorous shakes. If the conditions are right, hardly a single air-bubble will form as a result of the shaking. Much oxalate or citrate interferes with the coagulation and later with the uric acid determination; 20 mg. of potassium oxalate is ample for 10 c.c. of blood. Citrate, except in the minimum amount, is to be avoided. When the blood is properly coagulated, the color of the coagulum gradually changes from pink to dark brown. If this change does not occur, the coagulation is incomplete, due to too much oxalate or citrate. In such an emergency the sample may be saved by adding 2 normal sulphuric acid drop by drop, shaking vigorously after each addition and allowing the mixture to stand for a few minutes before adding more, until the coagulation is complete. Pour the mixture on a filter large enough to hold the entire contents of the flask and cover with a watch glass. If the filtration is begun by pouring the first few c.c. of the mixture down the double portion of the

filter paper and withholding the remainder until the whole filter has been wet, the filtrates are almost invariably as clear as water from the first drop. If a filtrate is not perfectly clear, the first 2 or 3 c.c. must be returned to the funnel. (Filter papers of the following diameters will meet all ordinary needs; 11, 12½, 15 and 18½ cm.)

In the precipitation of the blood protein, a special blood pipette is employed. This is simply a 15 c.c. pipette, graduated from the long tip into 1 c.c. portions. It is convenient to use three such pipettes, one for the blood, one for the sodium tungstate solution, and one for the sulphuric acid. The water used for diluting the blood may be measured with a cylinder.

For the proper carrying through of this thod it is essential that suitable sodium tungstate be employed. If the tungstate contains too much carbonate, the portion of the sulphuric acid will be used up and the lack of sulphuric acid will cause a loss in the later determination of uric acid. A safe and convenient criterion is to test the blood filtrate obtained with Congo red paper. The reaction should be negative or at the most just perceptible.

The carbonate content of sodium tungstate is easily determined as follows: To 10 c.c. of 10 per cent solution add 1 drop of phenolphthalein and titrate with 0.1 normal hydrochloric acid. Each c.c. of hydrochloric acid corresponds to 1.06 per cent of sodium carbonate. The amount of acid required for the titration should not exceed 0.4 c.c.

The blood filtrates are nearly neutral, 10 c.c. of filtrate requiring only about 0.2 c.c. of 0.1 normal sodium hydroxide when titrated with phenolphthalein as indicator. If the filtrates are to be kept for any length of time, more than two or three days, they need some preservation. One or 2 drops of toluene or xylene is adequate for the filtrate obtained from 10 c.c. of blood. This method is applicable to all kinds of blood, for example, human, beef, sheep, chicken, dog and rabbit.

DETERMINATION OF NON-PROTEIN NITROGEN

Introduce 5 c.c. of protein-free blood filtrates into a dry 75 c.c. test-tube graduated at 35 c.c. and at 50 c.c. Add 1 c.c. of the sulphuric-phosphoric acid mixture. Add a dry quartz pebble and boil vigorously over a micro-burner until the characteristic dense

acid fumes begin to fill the test-tube. This is usually accomplished in from three to seven minutes. When the fumes are unmistakable, cut down the size of the flame so that the contents of the tube are just visibly boiling, and close the mouth of the test-tube with a watch glass or a very small Erlenmeyer flask. Continue the heating very gently for two minutes from the time the fumes began to be unmistakable, even if the solution has become clear and colorless at the end of twenty to forty seconds. If the oxidations are not visibly finished at the end of two minutes, the heating must be continued until the solution is nearly colorless. Such cases are very rare; the oxidation is almost invariably finished within the first minute. Allow the contents to cool from seventy to ninety seconds and then add 15 to 25 c.c. of water. Cool further, approximately to room temperature, and add water to the 35 c.c. mark. Add, preferably with a pipette, 15 c.c. of the special Nessler solution. Insert a clean rubber stopper and mix. If the solution is turbid, centrifuge a portion before making the color comparison with the standard. The standard most commonly required is 0.3 mg. of nitrogen (in the form of ammonium sulphate) in a 100 c.c. flask. Add to it 2 c.c. of the sulphuric-phosphoric acid mixture, about 50 c.c. of water, and 30 c.c. of Nessler solution. Fill to the mark and mix. The unknown and the standard should be Nesslerized at approximately the same time. If the standard is set at 20 mm. for the color comparison, 20 divided by the reading and multiplied by 30 gives the non-protein nitrogen in mg. for 100 c.c. of blood.

Preparation of Reagents for Non-protein Nitrogen Method

Sulphuric-phosphoric Acid Solution.—Mix 300 c.c. of phosphoric acid, syrup (about 85 per cent H_3PO_4) with 100 c.c. of concentrated sulphuric acid. Transfer to a tall cylinder, cover well to exclude the absorption of ammonia, and set aside for sedimentation of calcium sulphate. This sedimentation is very slow, but in the course of a week or so the top part is clear and 50 to 100 c.c. can be removed by means of a pipette. (It is not absolutely necessary that the calcium should be thus removed, but it is probably a little safer to have it done.) To 100 c.c. of the clear acid add 10 c.c. of 6 per cent copper sulphate solution and 100 c.c. of water.

Nessler Solution.—From completely saturated caustic soda solution, containing about 55 gms. of sodium hydroxide per 100 c.c., decant the clear supernatant liquid and dilute to a concentration of 10 per cent. (It is worth while to determine by titration that a 10 per cent solution has been obtained within an error of not over 5 per cent.) Introduce into a large bottle 3500 c.c. of 10 per cent sodium hydroxide solution, add 750 c.c. of mercuric potassium iodide solution, to be described below, and 750 c.c. of distilled water, giving 5 liters of Nessler solution.

The Nessler solution so obtained contains enough alkali in 15 c.c. to neutralize 1 c.c. of the diluted sulphuric acid mixture and to give a suitable degree of alkalinity for the development of the color given by ammonia at a volume of 50 c.c. (In other Nesslerizations, as in urine analysis, when there is no acid to be neutralized 10 c.c. of the Nessler reagent per 100 c.c. of Nesslerized ammonia solution is the correct amount.)

Mercuric Potassium Iodide Solution.—Transfer 150 gms. of potassium iodide and 110 gms. of iodine to a 500 c.c. Florence flask; add 100 c.c. of water and an excess of metallic mercury, 140 to 150 gms. Shake the flask continuously and vigorously for seven to fifteen minutes or until the dissolved iodine has nearly disappeared. The solution becomes quite hot. When the red iodine solution has begun to become visibly pale, though still red, cool in running water and continue the shaking until the reddish color of the iodine has been replaced by the greenish color of the double iodide. The whole operation usually does not take more than fifteen minutes. Now separate the solution from the surplus mercury by decantation and washing with liberal quantities of distilled water. Dilute the solution and washings to a volume of 2 liters. If the cooling is begun in time, the resulting reagent is clear enough for immediate dilution with 10 per cent alkali and water, and the finished solution can at once be used for Nesslerizations.

DETERMINATION OF UREA

Principle.—The principle of this method consists in the hydrolysis of urea by means of urease or heat and the isolation of the ammonia by aeration or distillation. It is thus evident that there are four combinations, any one of which will give satisfactory results.

1. Determination of Urea by Urease and Distillation

Procedure.—Transfer 5 c.c. of the tungstic acid blood filtrate to a *clean* and *dry* Pyrex ignition tube (capacity about 75 c.c.). The graduated Pyrex tubes recommended for the non-protein nitrogen determinations should never be used for urea determinations, because they have contained Nessler solutions and Nessler solutions leave behind films of mercury compounds which destroy the urease. If these tubes must be used, they should first be washed with nitric acid to remove the mercury films and then thoroughly washed with water. Add to the blood filtrate 2 drops of the pyrophosphate solution or 2 drops of a molecular *o*-phosphate solution (1/3 molecular monosodium phosphate plus 2/3 molecular disodium phosphate). Then add 0.5 to 1 c.c. of the urease solution and immerse the test-tube in a beaker of warm water and leave it there for five minutes. The temperature of the water is not very important but should not exceed 55° C. Warm water can perhaps scarcely be said to be essential, for the hydrolysis is very rapid at room temperature, but it is preferable to use it. If no hot water is used, continue the digestion for ten to fifteen minutes or as much longer as is convenient. The ammonia formed can be conveniently and quickly distilled into 2 c.c. of 0.05 normal hydrochloric acid contained in the second test-tube. The second test-tube should not be so heavy as the ordinary test-tubes and should be graduated at 25 c.c. The test-tube which serves as the receiver is held in place by means of a rubber stopper in the side of which has been cut a fairly deep notch to permit the escape of air (and some steam). The rubber stopper serving as a holder for the receiver fits quite loosely to the delivery tube by means of which the two test-tubes are connected. The delivery tube must, of course, be so adjusted as to reach below the surface of the hydrochloric acid solution in the receiver before the distillation is begun.

Add to the hydrolyzed blood filtrate a dry pebble, 2 c.c. of saturated borax solution, and a drop or two of paraffin oil; insert firmly the rubber stopper carrying both delivery tube and receiver, and boil moderately fast over a micro-burner for four minutes. The size of the flame should never be cut down during the distillation nor should the boiling be so brisk that the emission of steam from the receiving tube begins before the end of three minutes. At the end of four minutes slip off the receiver from the

rubber stopper and draw the connecting tube diagonally to near 1/3 the top of the inclined receiver. Continue the distillation for one more minute and rinse off the lower outside part of the delivery tube with a little water. Cool the distillate with running water, dilute to about 20 c.c. and add 2.5 c.c. of the Nessler solution previously described. Fill to the 25 c.c. mark and compare in the colorimeter with a standard containing 0.3 mg. of nitrogen in a 100 c.c. flask and Nesslerized with 10 c.c. of the Nessler solution. The standard and unknown should always be Nesslerized as nearly simultaneously as practicable.

Calculation.—Multiply 20 (the height of the standard in mm.) by 15 and divide by the colorimetric reading to get the urea nitrogen per 100 c.c. of blood. The reasons for this calculation are, of course, to be found in the fact that the standard containing 0.3 mg. of nitrogen is diluted to 100 c.c. while the unknown, which corresponds to 0.05 c.c. of blood, is diluted to only 25 c.c.

Preparation of Urease.—Urease solution is made from jack bean powder in the following manner: Transfer to a 200 c.c. flask or bottle about 3 gms. of permutit powder. Wash this by decantation once with 2 per cent acetic acid, then twice with water. Add to the moist permutit in the flask 100 c.c. of 30 per cent alcohol (35 c.c. of 95 per cent alcohol mixed with 70 c.c. of water). First introduce 5 gms. of jack bean meal and shake for ten minutes. Filter and collect the filtrate in three or four different clean, small bottles. Set one aside for immediate use; it will remain serviceable for at least one week at ordinary room temperature if not exposed to direct sunlight. Put the others on ice where they will remain good from three to five weeks. This preparation is very active and the use of permutit makes the extract free from ammonia, nor does ammonia develop on standing.

Preparation of Pyrophosphate Solution.—This solution contains 140 gms. of sodium pyrophosphate (U.S.P.) and 20 gms. of glacial phosphoric acid per liter.

2. Urea Determination by Means of Urease and Aeration

The decomposition of the urea is made in the same kind of a Pyrex test-tube and in the manner already described under 1. One or 2 c.c. of 10 per cent sodium hydroxide are first added and ammonia aspirated into a test-tube graduated at 25 c.c. and con-

taining 2 c.c. of 0.05 normal hydrochloric acid. The rest of the process is as described under 1. The only precaution likely to be overlooked is that the rubber tubing used for connections needs to be rinsed with water before being used the first time, and, later also, if the tubing has been idle for any length of time. The talcum powder with which the inner and outer surface of rubber tubing is coated is probably the source of the trouble in the case of new rubber tubing. It is probably contaminated with ammonia.

3 and 4. Urea Determination by Means of Autoclave Decomposition

To 5 c.c. of blood filtrate in a 75 c.c. test-tube is added 1 c.c. of normal acid; the mouth of the test-tube is covered with tin-foil, and the test-tube with contents is then heated in the autoclave at 150° C., for ten minutes.

Allow the autoclave to cool to below 100° before opening. The ammonia is then distilled off exactly as in the first process described, except that 2 c.c. of 10 per cent sodium carbonate are substituted for the borax, or the ammonia may be removed by aeration as described in the second method.

DETERMINATION OF PREFORMED CREATININE

Principle.—The same as that for the determination of creatinine in urine.

Procedure.—Transfer 25 (or 50) c.c. of a saturated solution of purified picric acid to a small clean flask, add 5 (or 10) c.c. of 10 per cent sodium hydroxide, and mix. Transfer 10 c.c. of blood filtrate to a small flask or to a test-tube, transfer 5 c.c. of the standard creatinine solution, to be described below, to another flask, and dilute the standard to 20 c.c. Then add 5 c.c. of the freshly prepared alkaline picrate solution to the blood filtrate, and 10 c.c. to the diluted creatinine solution. Let stand for eight to ten minutes and make the color comparison in the usual manner, never omitting first to ascertain that the two fields of the colorimeter are clear when both cups contain the standard creatinine picrate solution. The color comparison should be completed within fifteen minutes from the time the alkaline picrate was

added; it is, therefore, never advisable to work with more than 3 to 5 blood filtrates at a time.

When the amount of blood filtrate available for the creatinine determination is too small to permit repetition, it is, of course, advantageous or necessary to start with more than one standard. If the high creatinine should be encountered unexpectedly without several standards ready, the determination can be saved by diluting the unknown with an appropriate amount of the alkaline picrate solution—using for such dilution the picrate solution first diluted with 2 volumes of water—so as to preserve equality between the standard and the unknown in relation to the concentration of picric acid and sodium hydroxide.

Preparation of Standard Creatinine Solution.—One standard creatinine solution, suitable for both creatinine and creatine determinations in blood, can be made as follows: Transfer to a liter flask 6 c.c. of the standard creatinine solution used for urine analysis (which contains 6 mg. of creatinine); add 10 c.c. of normal hydrochloric acid, dilute to the mark with water, and mix. Transfer to a bottle and add 4 or 5 drops of toluene or xylene. Five c.c. of this solution contain 0.03 mg. of creatinine, and this amount plus 15 c.c. of water represents the standard needed for the vast majority of human bloods, for it covers the range of 1 to 2 mg. per 100 c.c. In the case of unusual bloods representing retention of creatinine, take 10 c.c. of the standard plus 10 c.c. of water, which covers the range of 2 to 4 mg. of creatinine per 100 c.c. of blood; or 15 c.c. of the standard plus 5 c.c. of water, by which 4 to 6 mg. can be estimated. By taking the full 20 c.c. volume from the standard solution at least 8 mg. can be estimated; but when working with such blood it is well to consider whether it may not be more advantageous to substitute 5 c.c. of blood filtrate plus 5 c.c. of water for the usual 10 c.c. of blood filtrate.

Calculation.—The reading of the standard in mm. (usually 20) multiplied by 1.5, 3, 4.5, or 6 (according to how much of the standard solution was taken), and divided by the reading of the unknown, in mm., gives the amount of creatinine, in mg. per 100 c.c. of blood. In connection with this calculation it is to be noted that the standard is made up to twice the volume of the unknown, so that each 5 c.c. of the standard creatinine solution, while containing 0.03 mg., corresponds to 0.015 mg. in the blood filtrate.

DETERMINATION OF CREATINE PLUS CREATININE

Principle.—The principle is the same as that employed in the determination of creatine in the urine.

Procedure.—Transfer 5 c.c. of blood filtrate to a test-tube graduated at 25 c.c. These test-tubes are also used for urea and for sugar determinations. Add 1 c.c. of normal hydrochloric acid. Cover the mouth of the test-tube with tin-foil and heat in the autoclave to 130° C., for twenty minutes, or, as for the urea hydrolysis to 155° C., for ten minutes. Cool. Add 5 c.c. of the alkaline picrate solution and let stand for eight to ten minutes, then dilute to 25 c.c. The standard solution required is 20 c.c. of creatinine solution in a 50 c.c. volumetric flask. Add 2 c.c. of normal acid and 10 c.c. of the alkaline picrate solution and after ten minutes standing dilute to 50 c.c. The preparation of the standard must, of course, have been made first so that is it ready for use when the unknown is ready for the color comparison. The height of the standard, usually 20 mm., divided by the reading of the unknown and multiplied by 12 gives the "total creatinine" in mg. per 100 c.c. of blood.

In the case of uremic bloods containing large amounts of creatinine 1, 2, or 3 c.c. of blood filtrate, plus water enough to make approximately 5 c.c. are substituted for 5 c.c. of undiluted filtrate.

The normal value for "total creatinine" given by this method is about 6 mg. per 100 c.c. of blood.

DETERMINATION OF URIC ACID

Principle.—Uric acid is separated from the blood filtrate as the silver salt. The silver urate is then dissolved by means of alkaline sodium cyanide solution. By means of a uric acid reagent a color is developed the depth of which is compared to a standard solution of uric acid to which has been added the uric acid reagent.

Solutions Required for Uric Acid Determinations.—1. *Standard uric acid sulphite solution* prepared as follows: Make 1 to 3 liters of a 20 per cent solution of sodium sulphite, let stand over night, and filter. Dissolve 1 gm. of uric acid in 125 to 150 c.c. of 0.4 per cent lithium carbonate solution, and dilute to a volume of 500 c.c. Transfer 50 c.c., corresponding to 100 mg. of uric acid, to each of

a series of volumetric liter flasks. Add 200 c.c. to 300 c.c. of water, then 500 c.c. of filtered 20 per cent sodium sulphite solution, and finally make up to volume, and mix well. Fill a series of 200 c.c. bottles, and stopper very tightly with rubber stoppers. The solution in a bottle which is opened daily will keep for at least three to four months. In unopened bottles the uric acid will probably keep for years. Of this solution 3 c.c. is to be used for each series of determinations.

2. *A 10 per cent sodium sulphite solution.* The surplus 20 per cent sulphite solution from the preparation of the standard uric acid sulphite solution should be diluted to a concentration of 10 per cent and then be transferred to a series of small, tightly stoppered bottles. This sulphite is added to the unknown in order to offset the sulphite content of the standard. Two c.c. of this solution are used for each determination.

3. *A 5 per cent sodium cyanide solution, to be added from a burette,* 2.5 to 5 c.c. used for each series of determinations.

4. *A 10 per cent solution of sodium chloride in 0.1 normal hydrochloric acid.* Ten to 20 c.c. to be used for each series of determinations.

5. *The uric acid reagent.* Boil together 100 gms. of sodium tungstate, 80 c.c. of phosphoric acid (85 per cent) and 700 c.c. of water for two hours under a reflux condenser. Dilute the solution to 1 liter.

6. *A solution of 5 per cent silver lactate in 5 per cent lactic acid.* Four to 5 c.c. of this solution are needed for each determination.

Procedure.—To 10 c.c. of blood filtrate in each of two centrifuge tubes (capacity 15 c.c.) or 20 c.c. of blood filtrate in 30 c.c. test-tubes, add 2 c.c. of a 5 per cent solution of silver lactate in 5 per cent lactic acid and stir with a very fine glass rod. Centrifuge; add a drop of silver lactate to the supernatant solution which should be almost perfectly clear and should not become turbid when the last drop of silver solution is added. Remove the supernatant liquid by decantation as completely as possible. Add to each tube 1 c.c. of a solution of 10 per cent sodium chloride in 0.1 normal hydrochloric acid and stir thoroughly with a glass rod. Then add 5 to 6 c.c. of water, stir again, and centrifuge once more. By this chloride treatment the uric acid is set free from the precipitate. Transfer the two supernatant liquids by decantation to a 25 c.c. volumetric flask. Add 1 c.c. of a 10 per cent solution of

sodium sulphite, 0.5 c.c. of a 5 per cent solution of sodium cyanide, and 3 c.c. of a 20 per cent solution of sodium carbonate. Prepare simultaneously two standard uric acid solutions as follows:

Transfer to one 50 c.c. volumetric flask 1 c.c. and to another 50 c.c. flask 2 c.c. of the standard uric acid sulphite solution described above. To the first flask add also 1 c.c. of 10 per cent sodium sulphite solution. Then add to each flask 4 c.c. of the acidified sodium chloride solution, 1 c.c. of the sodium cyanide solution, and 6 c.c. of the sodium carbonate solution. Dilute with water to about 45 c.c. When the two standard solutions and the unknown have been prepared as described, they are ready for the addition of the uric acid reagent. Add 0.5 c.c. of this reagent to the unknown and 1 c.c. to each of the standards, and mix. Let stand for ten minutes, fill to the mark with water, mix, and make the color comparison.

Calculation.—In connection with the calculation it is to be noted (*a*) that the blood filtrate taken corresponds to 2 c.c. of blood, (*b*) that the standard is diluted to twice the volume of the unknown, and (*c*) that the standard used contains 0.1 or 0.2 mg. of uric acid. The blood filtrate from blood containing 2.5 mg. of uric acid will be just equal in color to the weaker standard. Twenty times 2.5 divided by the reading of the unknown, gives, therefore, the uric acid content of the blood when the weaker standard is set at 20 mm.

Remarks.—At times the uric acid content may sink to as low as 1 mg. of uric acid per 100 c.c. of blood. A standard corresponding to the color obtained from 1.25 mg. of uric acid per 100 c.c. of blood can be quickly prepared as follows:

Transfer 1 c.c. of 10 per cent sulphite solution, 3 c.c. of 20 per cent sodium carbonate, 2 c.c. of the acidified sodium chloride, 0.5 c.c. of the sodium cyanide solution, and 25 c.c. of the weaker one of the two regular standard solutions already on hand. Dilute to 50 c.c. and mix. Or, simply add 5 c.c. of 20 per cent sodium carbonate to 25 c.c. of the regular weaker standard, and dilute to 50 c.c.

If a low uric acid value is expected, an alternate procedure is to dilute the unknown to a final volume of 10 c.c. with corresponding reduction in the amount of the reagents used.

The uric acid reagent must invariably be added after, and not before, the addition of the sodium carbonate, because in acid

solution the sulphite will itself give a blue color with the phosphotungstic acid.

DETERMINATION OF SUGAR

Method of Folin and Wu[1]

Principle.—When sugar and an alkaline solution of copper are heated cuprous oxide is formed. In the presence of a molybdate phosphate solution cuprous oxide gives rise to an intense and stable color reaction. The color so obtained is compared with the color given by a standard solution of sugar.

Solutions Needed. 1. *Standard Sugar Solution.*—Three standard sugar solutions should be on hand: (1) a stock solution, 1 per cent dextrose or invert sugar, preserved with xylene or toluene; (2) a solution containing 1 mg. of sugar per 10 c.c. (5 c.c. of stock solution diluted to 500 c.c.); (3) a solution containing 2 mg. of sugar per 10 c.c. (5 c.c. of the stock solution diluted to 250 c.c.) The diluted solutions should be preserved with a little added toluene or xylene; it is better not to depend on such diluted solutions to keep for more than a month, but the stock solution should keep indefinitely. If pure dextrose is not available, a standard solution of invert sugar may be made from cane sugar and is equally useful. To make this transfer exactly 1 gm. of cane sugar to a 100 c.c. volumetric flask; add 20 c.c. of normal hydrochloric acid and let the mixture stand over night at room temperature (or rotate the flask and contents continuously in the water bath at 70° C.). Add 1.68 gm. of sodium bicarbonate and about 0.2 gm. of sodium acetate to neutralize the hydrochloric acid. Shake a few minutes to remove most of the carbonic acid and fill to the 100 c.c. mark with water. Then add 5 c.c. more of water (1 gm. of cane sugar yields 1.05 gms. of invert sugar) and mix. Transfer to a bottle; add a few drops of xylene or toluene, shake well, and stopper tightly.

2. *Alkaline Copper Solution.*—Dissolve 40 gms. of anhydrous sodium carbonate in about 400 c.c. of water and transfer to a liter flask. Add 7.5 gms. of tartaric acid and when the latter has dissolved add 4.5 gms. of crystallized copper sulphate; mix and make up to a volume of 1 liter. If the carbonate used is impure a sedi-

[1] Folin and Wu: Jour. Biol. Chem., 1920, 41, 367.

ment may be formed in the course of a week or so. If this happens, decant the clear solution into another bottle.

3. *Molybdate Phosphate Solution.*—Transfer to a liter beaker 35 gms. of molybdic acid and 5 gms. of sodium tungstate. Add 200 c.c. of 10 per cent sodium hydroxide and 200 c.c. of water. Boil vigorously for twenty to forty minutes so as to remove nearly the whole of the ammonia present in the molybdic acid. Cool, dilute to about 350 c.c. and add 125 c.c. of concentrated (85 per cent) phosphoric acid. Dilute to 500 c.c.

Procedure.—Heat a beaker of water to vigorous boiling. Transfer 2 c.c. of the tungstic acid blood filtrate to a *blood sugar test-tube*, and to two other similar test-tubes (graduated at 25 c.c.) add 2 c.c. of standard sugar solution containing respectively 0.2 and 0.4 mg. of dextrose. To each tube add 2 c.c. of the alkaline copper solution. The surface of the mixtures must now have reached the constricted part of the tube. If the bulb of the tube is too large for the volume (4 c.c.) a little, but not more than 0.5 c.c. of a diluted (1:1) alkaline copper solution may be added. If this does not suffice to bring the contents to the narrow part, the tube should be discarded. Test-tubes having so small a capacity that 4 c.c. fills them above the neck should also be discarded. Heat the tubes in a boiling water bath for six minutes. Then transfer them to a cold water bath and let cool, without shaking, for two to three minutes. Add to each test-tube 2 c.c. of the molybdate phosphate solution. The cuprous oxide dissolves rather slowly if the amount is large but the whole, up to the amount given by 0.8 mg. of dextrose, dissolves easily within two minutes. When the cuprous oxide is dissolved, dilute the resulting blue solutions to the 25 c.c. mark, insert a rubber stopper, and mix. It is essential that adequate attention be given to this mixing because the greater part of the blue color is formed in the bulb of the tube. At least five minutes after mixing make the color comparison in the usual manner.

Calculation.—The depth of the standard (in mm.) multiplied by 100 and divided by the reading of the unknown gives the sugar content, in mg., per 100 c.c. of blood.

Remarks.—The two standards given representing 0.2 and 0.4 mg. of glucose are adequate for practically all cases. They cover the range from 70 to nearly 400 mg. of glucose per 100 c.c. of blood.

TOTAL NITROGEN

The total nitrogen of the blood may be readily determined by the regular Kjeldahl method. One c.c. of the blood accurately measured is used in this method.

AMMONIA IN THE BLOOD

Method of Folin and Denis [1]

Principle.—Same as for ammonia in urine.

Procedure.—10 c.c. of systemic blood or 5 c.c. portal or mesenteric blood are transferred to a suitable large test-tube. To it are added 2 to 3 c.c. of an oxalate carbonate solution (15 per cent potassium oxalate and 10 per cent sodium carbonate) and about 5 c.c. toluene. The air current is then connected and the ammonia is carried over into a standard acid solution. The current is run as fast as possible for twenty to thirty minutes. The ammonia thus collected is Nesslerized in the usual manner for blood nitrogen.

Note.—Ammonia determinations in the blood are not accurate inasmuch as blood decomposes spontaneously (especially in the presence of alkalies capable of setting free the ammonia) at all temperatures even when kept on ice.

UREA

The Urease Method, Van Slyke and Cullen's [2] *Modification of Marshall's Method* [3]

Principle.—See Urease Method, page 38.

Procedure.—Run 3 c.c. of fresh blood (carefully measured with an accurate pipette) into a 100 c.c. test-tube containing 1 c.c. of a 3 per cent solution of potassium citrate (to prevent clotting). Add 0.5 c.c. of the urease solution [4] and 2 or 3 drops of caprylic alcohol (to prevent foaming). After ten minutes add 15 c.c. of a saturated solution of potassium carbonate, and drive off the

[1] Folin and Denis: Jour. Biol. Chem., 1912, 11, 532.

[2] Van Slyke and Cullen: J. Am. Med. Assn., 1914, 62, 1558.

[3] Marshall: Jour. Biol. Chem , 1913, 15, 487.

[4] The enzyme solution is prepared as described on page 89.

ammonia by aspiration into another tube (Van Slyke and Cullen apparatus, page 39 may be used) containing 15 c.c. of hundredth-normal hydrochloric or sulphuric acid. Titrate the excess of acid with hundredth-normal sodium hydroxide or potassium hydroxide, using methyl red or alizarin as indicator.

Calculations.—Each cubic centimeter of acid neutralized indicates 0.01 gram of urea per 100 c.c. of blood, or 0.00467 gm. of urea nitrogen per 100 c.c. of blood. In case the blood should be one of the rare samples containing over 0.15 per cent of urea, all the acid will be neutralized, and it will be necessary to repeat the determinations, using in the determination only 1 c.c. of blood. Fresh blood contains so little ammonia that it may be disregarded.

URIC ACID

Benedict's Method [1]

Principle.—Removal of protein from the blood by coagulation and addition of colloidal iron, and the colorimetric estimation of uric acid in the filtrate.

Procedure.—To 100 c.c. of boiling N/100 acetic acid in a casserole, 20 c.c. [2] of oxalated blood are added, and the mixture is heated to boiling for a moment. Remove the casserole from the flame and add 200 c.c. of boiling distilled water. Pour the mixture upon a folded filter and wash the residue with 50 c.c. of boiling water (heated in the same casserole in which the original coagulation took place). Transfer the whole filtrate to a casserole and boil down rapidly to a volume of about 25 c.c. Pour this solution into a small flask roughly marked to indicate a volume of 50 c.c. Transfer the contents of the casserole to the flask quantitatively, with the help of two or three portions of water, heating vigorously to boiling and rubbing the sides of the casserole with a rubber-tipped stirring rod each time. The total volume in the flask should not exceed 50 c.c. after the addition of the washings. Thoroughly cool the turbid solution in the flask under

[1] Benedict: Jour. Biol. Chem., 1915, 20, 629.

[2] Smaller amounts of blood may be employed, and the quantity of acetic acid and water correspondingly reduced. Unless the quantity of uric acid present is very large, the results are far more accurate when 20 c.c. of blood are used.

running water, and add 2 c.c.[1] of colloidal iron solution (Merck's "Dialyzed Iron," 5 per cent solution) while the flask is being gently rotated. Filter the mixture through a small folded filter into a 100 c.c. Jena Florence flask, and wash the residue twice with distilled water. The filtrate obtained here should be as clear and colorless as distilled water. Boil the solution down to a volume of from 1 to 2 c.c. (care being taken in the early stages to prevent bumping), then carefully pour into a small centrifuge tube and wash out the flask with three portions of water (1 or 2 c.c. each), heating each to boiling in the flask and shaking thoroughly prior to transferring it to the centrifuge tube. The volume of liquid in the tube at this point should be from 5 to 10 c.c. Cool the liquid, add 20 drops of the ammoniacal silver magnesium solution. Mix the contents of the tube with a small stirring rod and centrifuge the tube for one or two minutes. Pour off the supernatant liquid, as completely as possible, by inverting the tube, allowing it to drain a moment, and then touching the inside of the lip of the tube with a towel or piece of filter paper. Add to the residue in the tube two drops of a 5 per cent solution of potassium cyanide to dissolve the silver urate, stir the mixture thoroughly with a thin rod, for half a minute, add a few drops (0.5 to 1.0 c.c.) of water, and stir again.[2] Two c.c. of the uric acid reagent are added and the mixture stirred again, after which[3] add 10 c.c. of 20 per cent sodium carbonate solution, transfer quantitatively to a 50 c.c. flask, and at the end of about one-half minute, dilute to mark. Compare this solution in the Duboscq colorimeter with a simultaneously prepared solution obtained by treating 5 c.c. of the standard uric acid solution, contained in a 50 c.c. flask, with 2 drops of the potassium cyanide solution, 2 c.c. of the uric acid

[1] With old samples of blood it may be necessary to add 3 or 4 c.c. of the iron solution and a little 10 per cent sodium chloride solution. When the precipitate separates in large flocculent masses the right amount of iron has been added. Any excess of iron must be avoided, as it would oxidize some of the uric acid later on in the process.

[2] At this point perfectly clear solutions are obtained with pure uric acid solutions in phosphate mixture or in pyridin. With urines some magnesium ammonium phosphate is precipitated with the uric acid, which does not dissolve in the cyanide. After adding the two subsequent reagents, however, a perfectly clear solution is obtained.

[3] Bogert (Jour. Biol. Chem., 1917, 31, 165) advocates transferring the mixture to the volumetric flask by washing with 20 to 30 c.c. of water before adding the sodium carbonate, as a means of preventing turbidity.

reagent, 10 c.c. of 20 per cent sodium carbonate solution, and diluting to the mark at the end of about one-half minute. The standard solution is best set at a height of 15 mm. in the colorimeter.

Calculation.—The reading of the standard divided by the reading of the urine gives the number of milligrams of uric acid in the amount of sample taken.

If the amount of uric acid present is very small the addition of 1 drop of cyanide solution, 1 c.c. of uric acid reagent, 5 c.c. of 20 per cent sodium carbonate solution, and dilution to 25 c.c. are carried out rather than using the larger quantities given for the determination in the urine.

Preparation of Solutions. *Ammoniacal Silver Magnesium Solution.*—This solution has the following composition:

3 per cent silver lactate solution	70 c.c.
Magnesia mixture	30 c.c.
Concentrated ammonium hydroxide solution	100 c.c.

Uric Acid Reagent.—Boil together 100 gms. of sodium tungstate, 30 c.c. of 85 per cent phosphoric acid, 20 c.c. of concentrated hydrochloric acid, and 750 c.c. of distilled water under a reflux condenser for 1½ hours. Cool and make up to 1 liter. This modified solution shows less tendency to develop turbidity than does the original Folin-Denis reagent.

Sodium Carbonate Solution.—Dissolve 200 gms. of anhydrous sodium carbonate in warm water and make up to 1 liter.

Standard Uric Acid Solution.—The solution of uric acid in phosphate solution is very readily prepared, does not need to be standardized, and appears to keep indefinitely. It is prepared in the following manner: Dissolve 9 gms. of pure crystallized disodium hydrogen phosphate, together with 1 gm. of crystallized sodium dihydrogen phosphate, in 200 to 300 c.c. of hot water, and filter if the solution is not perfectly clear. Make this filtrate up to about 500 c.c. with hot water, and pour this hot or warm (and perfectly clear) solution upon exactly 200 mg. of pure uric acid suspended in a few cubic centimeters of water in a liter volumetric flask. Agitate the mixture for a few minutes until the uric acid completely dissolves. Cool, add *exactly* 1.4 c.c. of glacial acetic acid, dilute to the mark, and mix. Add about 5 c.c. of chloroform to

prevent the growth of bacteria or moulds in the solution. Five c.c. of this solution contains exactly 1 mg. of uric acid.

CREATINE AND CREATININE

Methods of Folin[1]

Preformed Creatinine.—Measure 10 c.c. of blood into a 50 c.c. volumetric flask or, better, into a 50 c.c. shaking cylinder which can be closed with a glass stopper. Fill to the 50 c.c. mark with saturated picric acid solution and shake a few times. Add about 1 gm. of dry picric acid to the mixture and shake for five minutes. Transfer the mixture to centrifuge tubes, throw down the sediment and precipitate and pour the supernatant liquid through a filter. This is the most economical process where but little blood is available. If desired, however, double quantities of blood and reagents may be taken and filtration carried out without preliminary centrifugation. This process removes the protein materials and leaves the creatine and creatinine in the filtrate which is a saturated picric acid solution. The preformed creatinine is then determined colorimetrically. For this purpose a standard solution of creatinine for comparison is necessary. This standard solution should contain 0.2 mg. of creatinine in 100 c.c. of saturated picric acid solution.

Take 20 c.c. portions each of the filtrate and of the standard solution. To each solution then add exactly 1 c.c. of 10 per cent NaOH from a burette. (If the blood filtrate become turbid on addition of alkali it must be centrifuged or filtered.) Allow to stand for ten minutes and compare the colors directly in the colorimeter without further dilution. The standard creatinine solution may be set advantageously at 20 mm., although this is not necessary.

Calculation.—Since the blood was diluted five times in the precipitation procedure and as the standard for comparison contains 0.2 mg. of creatinine per 100 c.c., it is merely necessary to divide the reading of the standard by the unknown to obtain without further calculation the number of milligrams of creatinine in 100 c.c. of blood.

[1] Folin: Jour. Biol. Chem., 1914, 17, 475.

Creatine Plus Creatinine.—For determining the total creatinine plus creatine in the blood carry out the preliminary precipitation with picric acid just as in the determination of creatinine above. Take 10 c.c. of this filtrate for the determination. Transfer it to a small Erlenmeyer flask or large test-tube. Cover the flask or test-tube with tin foil, transfer to an autoclave and heat to about 120° C. for about twenty minutes. The autoclave should not be opened until the temperature has fallen below 100° C. Cool the solution to room temperature, rinse into a 25 c.c. volumetric flask with saturated picric acid solution. Add 1.25 c.c. of 10 per cent NaOH for the development of the color.

On account of the variations in the creatine content of normal blood two standard creatinine solutions are used. In working on pathological cases a third standard is desirable. These standards contain 0.5, 1, and 2 mg. of creatinine respectively per 100 c.c. of saturated picric acid solution. To 20 c.c. of each of these solutions in measuring cylinders add 1 c.c. of 10 per cent NaOH and allow to stand for ten minutes. By inspection determine which standard corresponds most exactly in color with the unknown and use this for comparison. The standard is usually set at 10 mm. in the Duboscq colorimeter.

Calculation.—Multiply the reading of the standard by 125 and by 0.5, 1, or 2, according to which standard is used, and divide by the reading of the unknown in millimeters. The result gives the number of milligrams of creatine+creatinine in 100 c.c. of the blood examined.

AMINO-ACID NITROGEN

Method of Van Slyke and Meyer [1]

Principle.—The protein of the blood is removed by precipitation with alcohol and the amino-acid nitrogen determined in the filtrate by the nitrous acid method.

Procedure.—Thirty to 50 c.c. of freshly drawn blood are mixed with 9 to 10 volumes of 95 per cent alcohol to precipitate the proteins. The volume of the alcohol-blood mixture must be known, but in case it is not convenient to use a graduated cylinder for the mixture, its volume can be taken as the sum of the volumes

[1] Van Slyke and Meyer: Jour. Biol. Chem., 1912, 12, 399.

of the alcohol and blood without essentially affecting the results. The alcohol and blood are thoroughly mixed, the vessel containing them is closed and twenty-four hours are allowed for precipitation of the proteins to become complete. The solution is filtered through a dry folded filter into a measuring cylinder without washing the precipitate. The volume of filtrate is noted and is taken for analysis as an aliquot part of the total blood-alcohol mixture. The filtrate is then concentrated to a volume of 3 to 5 c.c. and used for determination of amino nitrogen by the Van Slyke nitrous acid method. The use of a few drops of caprylic alcohol to prevent foaming is advisable.

AMINO-ACID NITROGEN

Method of Greenwald and Bock [1]

Principle.—The protein of the blood is removed by heat coagulation and precipitation with trichloroacetic acid. The amino-acid nitrogen is determined in the filtrate by the nitrous acid method of Van Slyke.

Procedure.—If the blood is not over twenty-four hours old proceed as follows: Introduce into a flask approximately 0.4 gm. of ground (20 mesh) soy bean, 3 to 5 c.c. of water, and 1 c.c. of 3 per cent solution of crystallized NaH_2PO_4. Let stand a few minutes with occasional shaking. Run in 20 to 50 c.c. of blood and allow to stand at room temperature for thirty minutes. Heat 0.01 N acetic acid to boiling, using five volumes of acid for one of blood. Run in the blood slowly and boil, stirring for one-half minute. Add the same amount of boiling water and boil with stirring for 1 minute. Filter hot through a folded filter and wash the casserole three times with 30 c.c. portions of water, heating the water in the casserole and using a rubber-tipped stirring rod. Boil down the filtrate rapidly over a free flame in a casserole. Transfer to a small graduated flask or cylinder, choosing the size so as to obtain nearly the original volume of the blood. Wash the casserole three times with the smallest possible amount of water. The volume after washing should not be more than three-fourths of the final volume. Add enough trichloroacetic

[1] Greenwald and Bock: Jour. Biol. Chem., 1916–17, 28, 357.

acid to make an approximately 3 per cent solution (either the solid acid or a corresponding amount of 50 per cent solution is used). Dilute to volume, shake, and let stand thirty minutes. Add 2 gms. of kaolin, shake well, centrifuge, and filter. Transfer an aliquot part (15 to 40 c.c.) of the filtrate to a small flask (50 to 100 c.c.), add two or three beads, one drop of alizarin indicator, bring to a boil and keep slowly boiling (simmering) until the indicator turns. Add 1 to 2 c.c. of N potassium hydroxide, and boil one to two minutes to remove the ammonia. Make acid with a few drops of acetic acid and evaporate to a small volume. The whole amount may be used in the micro Van Slyke apparatus or the liquid transferred to a small graduated flask, diluted to a definite volume, and aliquot portions measured out by means of the burette of the Van Slyke apparatus.

If the blood is older than twenty-four hours, or if it has been laked or frozen, the proteins are precipitated according to the following procedure: A measured volume of blood (30 to 50 c.c.) is introduced into a flask which contains 0.4 gm. of ground soy bean, 3 to 5 c.c. of water, and 1 c.c. of a 3 per cent solution of NaH_2PO_4, and the mixture gently agitated. After standing for one-half hour at room temperature the mixture is precipitated by diluting to ten times its original volume with a 2.5 per cent solution of trichloroacetic acid. After standing for thirty minutes 2 gms. of kaolin are added, the mixture shaken vigorously, and filtered. An aliquot part of the filtrate is evaporated to a small volume, transferred to a small flask (50 to 100 c.c.) and treated from this point on as above.

THE QUANTITATIVE DETERMINATION OF ALIPHATIC AMINO GROUPS

Method of Van Slyke [1]

Principle.—This method for the determination of aliphatic amino nitrogen is based on the measurement of the nitrogen gas evolved in the reaction,

$$RNH_2 + HNO_2 = ROH + N_2 + H_2O.$$

[1] Van Slyke: Jour. Biol. Chem., 1912, 12, 275; 1913, 16, 121 and 125.

During the process the following reaction also takes place, the nitrous acid solution decomposing spontaneously with the formation of nitric oxide.

$$2HNO_2 = HNO_3 + NO.$$

This latter reaction is utilized in displacing all the air of the apparatus with nitric oxide. The amino solution is then introduced, evolution of nitrogen mixed with nitric oxide resulting. The oxide is absorbed with alkaline permanganate solution and the pure nitrogen measured in a special gas burette shown in the figure.

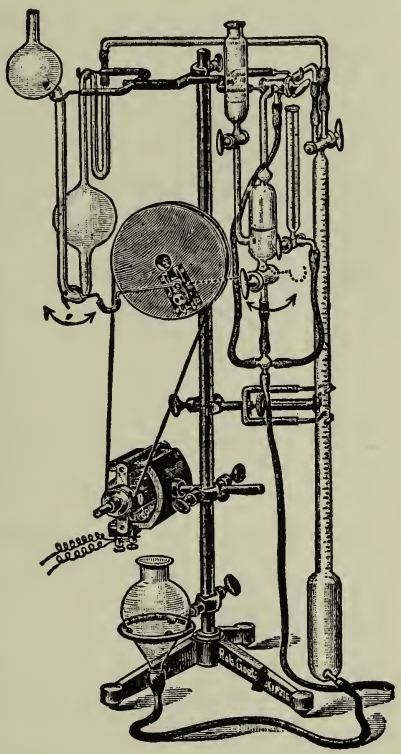

Fig. 2.—Van Slyke Amino Nitrogen Apparatus. (Hawk.)

Procedure.—The determination is carried out in three stages:

1. *Displacement of Air by Nitric Oxide.*—Water from *F* (see Figs. 2 and 3), fills the capillary leading to the Hempel pipette and also the other capillary as far as *c*. Into *A* one pours a volume of glacial acetic acid sufficient to fill one-fifth of *D*. For convenience, *A* is etched with a mark to measure this amount. The acid is run into *D*, cock *c* being turned so as to let the air escape from *D*. Through *A* one now pours sodium nitrite solution (30 gms. $NaNO_2$ to 100 c.c. H_2O) until *D* is full of solution and enough excess is present to rise a little above the cock into *A*. It is convenient to mark *A* for measuring off this amount also. The gas exit from *D* is now closed at *c*, and, *a* being open, *D* is shaken for a few seconds. The nitric oxide, which instantly collects, is let out at *c*, and the shaking repeated. The second crop of nitric oxide which washes

out the last portions of air, is also let out at *c*. *D* is now connected with the motor and shaken till all but 20 c.c. of the solution have been displaced by nitric oxide and driven back into *A*. A mark on *D* indicates the 20 c.c. point. One then closes *a* and turns *c* and *f* so that *D* and *F* are connected. The above manipulations require between one and two minutes.

2. *Decomposition of the Amino Substance.*—Of the amino solution to be analyzed 10 c.c. or less, as the case may be, are measured off in *B*. Any excess added above the mark can be run off through the outflow tube. The desired amount is then run into *D*, which is already connected with the motor, as shown in Fig. 2. It is shaken when α-amino acids are being analyzed for a period of three to five minutes. With α-amino acids, proteins, or partially or completely hydrolyzed proteins, we find that at the most five minutes' vigorous shaking completes the reaction. Only in the case of some native proteins which, when deaminized form unwieldy coagula that mechanically interfere with the thorough agitation of the mixture, a longer time may be required. In case a viscous solution is being analyzed and the liquid threatens to foam over into *F*, *B* is rinsed out and a little caprylic alcohol is added through it. For amino substances such as amino purins, requiring a longer time than five minutes to react, one merely mixes the reacting solutions and lets them stand the required length of time, then shakes about two minutes to drive the nitrogen completely out of solution.

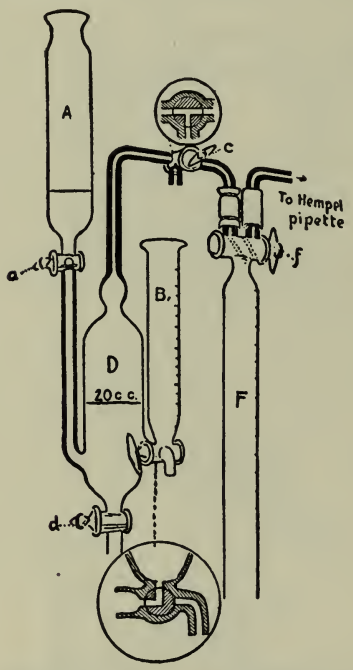

Fig. 3.—Section of Van Slyke Apparatus. (Hawk.)

When it is known that the solution to be analyzed is likely to foam violently, it is advisable to add caprylic alcohol through *B* before the amino solution. *B* is then rinsed with alcohol and dried

with ether or a roll of filter paper before it receives the amino solution.

3. *Absorption of Nitric Oxide and Measurement of Nitrogen.*— The reaction being completed, all the gas in *D* is displaced into *F* by liquid from *A* and the mixture of nitrogen and nitric oxide is driven from *F* into the absorption pipette (the solution in the absorption pipette is 40 gms. $KMnO_4$ and 25 gms. KOH in a liter). The driving rod is then connected with the pipette by lifting the hook from the shoulder of *d* and placing the other hook, on the opposite side of the driving rod, over the horizontal lower tube of the pipette. The latter is then shaken by the motor for a minute, which, with any but almost completely exhausted permanganate solutions, completes the absorption of nitric oxide. The pure nitrogen is then measured in *F*. During the above operations *a* is left open, to permit displacement of liquid from *D* as nitric oxide forms in *D*.

Blank determinations, performed as above except that 10 c.c. of distilled water replaces the solution of amino substances, must be performed on every fresh lot of nitrite used. Nitrite giving a much larger correction than 0.3 to 0.4 c.c. should be rejected.

The room temperature and the barometric pressure must be noted. The calculation of the weight of nitrogen gas corresponding to the volume obtained is most readily made with the aid of the tables (see page 108) devised for this purpose.

The Van Slyke Micro-apparatus.—In later work Van Slyke has used to a large extent an apparatus which differs from the one described above only in being considerably smaller. More accurate measurements can be made with this and smaller amounts of amino nitrogen determined. In using this only 10 c.c. of nitrite solution and 2.5 c.c. of acetic acid are required for an analysis. One-fifth the amount of substance may be analyzed with the same degree of accuracy as with the larger apparatus. Practically the only alteration from the mode of operation already detailed above, is in the speeds at which the deaminizing bulb and the Hempel pipette are shaken. During the first stage of the analysis the deaminizing bulb should be shaken by the motor at a very high rate of speed, about as fast as the eye can follow or an unnecessary amount of time is lost in freeing the apparatus from air. This stage is also much accelerated by warming the nitrite solution to 30° before it is used, in case a low room temperature has reduced the tem-

MILLIGRAMS OF AMINO NITROGEN CORRESPONDING TO 1 c.c. OF NITROGEN GAS AT 11° TO 30° C.; 728 TO 772 MM. PRESSURE

t	728	730	732	734	736	738	740	742	744	746	748	750	*t*
11°	0.5680	0.5695	0.5510	0.5725	0.5745	0.5760	0.5775	0.5790	0.5805	0.5820	0.5840	0.5855	11°
12°	0.5655	0.5670	0.5685	0.5700	0.5720	0.5735	0.5750	0.5765	0.5780	0.5795	0.5815	0.5830	12°
13°	0.5630	0.5645	0.5660	0.5675	0.5695	0.5710	0.5725	0.5740	0.5755	0.5770	0.5785	0.5805	13°
14°	0.5605	0.5620	0.5635	0.5650	0.5665	0.5680	0.5700	0.5715	0.5730	0.5745	0.5760	0.5775	14°
15°	0.5580	0.5595	0.5610	0.5625	0.5640	0.5655	0.5670	0.5685	0.5705	0.5720	0.5735	0.5750	15°
16°	0.5555	0.5570	0.5585	0.5600	0.5615	0.5630	0.5645	0.5660	0.5675	0.5690	0.5710	0.5725	16°
17°	0.5525	0.5540	0.5555	0.5575	0.5590	0.5605	0.5620	0.5635	0.5650	0.5665	0.5680	0.5695	17°
18°	0.5500	0.5515	0.5530	0.5545	0.5560	0.5580	0.5595	0.5610	0.5625	0.5640	0.5655	0.5670	18°
19°	0.5475	0.5490	0.5505	0.5520	0.5535	0.5550	0.5565	0.5580	0.5595	0.5610	0.5630	0.5645	19°
20°	0.5445	0.5460	0.5475	0.5495	0.5510	0.5525	0.5540	0.5555	0.5570	0.5585	0.5600	0.5615	20°
21°	0.5420	0.5435	0.5450	0.5465	0.5480	0.5495	0.5510	0.5525	0.5540	0.5555	0.5575	0.5590	21°
22°	0.5395	0.5410	0.5425	0.5440	0.5455	0.5470	0.5485	0.5500	0.5515	0.5530	0.5545	0.5560	22°
23°	0.5365	0.5380	0.5395	0.5410	0.5425	0.5440	0.5455	0.5470	0.5485	0.5500	0.5515	0.5530	23°
24°	0.5335	0.5350	0.5365	0.5380	0.5400	0.5415	0.5430	0.5445	0.5460	0.5475	0.5490	0.5505	24°
25°	0.5310	0.5325	0.5340	0.5355	0.5370	0.5385	0.5400	0.5415	0.5430	0.5445	0.5460	0.5475	25°
26°	0.5260	0.5295	0.5310	0.5325	0.5340	0.5355	0.5370	0.5365	0.5400	0.5415	0.5430	0.5445	26°
27°	0.5250	0.5265	0.5280	0.5295	0.5310	0.5325	0.5340	0.5355	0.5370	0.5385	0.5400	0.5415	27°
28°	0.5220	0.5235	0.5250	0.5265	0.5280	0.5295	0.5310	0.5325	0.5340	0.5355	0.5370	0.5385	28°
29°	0.5195	0.5210	0.5220	0.5235	0.5250	0.5265	0.5280	0.5295	0.5310	0.5325	0.5340	0.5355	29°
30°	0.5160	0.5175	0.5190	0.5205	0.5220	0.5235	0.5250	0.5265	0.5280	0.5295	0.5310	0.5325	30°
t	728	730	732	734	736	738	740	742	744	746	748	750	*t*

MILLIGRAMS OF AMINO NITROGEN CORRESPONDING TO 1 c.c. OF NITROGEN GAS AT 11° TO 30° C.; 728 TO 772 MM. PRESSURE—*Continued*

t	752	754	756	758	760	762	764	766	768	770	772	*t*
11°	0.5870	0.5885	0.5900	0.5915	0.5935	0.5950	0.5965	0.5980	0.5995	0.6010	0.6030	11°
12°	0.5845	0.5860	0.5875	0.5890	0.5905	0.5925	0.5940	0.5955	0.5970	0.5985	0.6000	12°
13°	0.5820	0.5835	0.5850	0.5865	0.5880	0.5895	0.5910	0.5930	0.5945	0.5960	0.5975	13°
14°	0.5790	0.5805	0.5825	0.5840	0.5855	0.5870	0.5885	0.5900	0.5915	0.5935	0.5950	14°
15°	0.5765	0.5765	0.5795	0.5810	0.5830	0.5845	0.5860	0.5875	0.5890	0.5905	0.5920	15°
16°	0.5740	0.5755	0.5770	0.5785	0.5800	0.5815	0.5830	0.5850	0.5865	0.5880	0.5895	16°
17°	0.5710	0.5730	0.5745	0.5760	0.5775	0.5790	0.5805	0.5820	0.5825	0.5850	0.5865	17°
18°	0.5685	0.5700	0.5715	0.5730	0.5745	0.5765	0.5780	0.5795	0.5810	0.5825	0.5840	18°
19°	0.5660	0.5675	0.5690	0.5705	0.5720	0.5735	0.5750	0.5765	0.5780	0.5795	0.5810	19°
20°	0.5630	0.5645	0.5660	0.5675	0.5690	0.5705	0.5725	0.5740	0.5755	0.5770	0.5785	20°
21°	0.5605	0.5620	0.5635	0.5650	0.5665	0.5680	0.5695	0.5710	0.5725	0.5740	0.5755	21°
22°	0.5575	0.5590	0.5605	0.5620	0.5635	0.5650	0.5665	0.5680	0.5695	0.5715	0.5730	22°
23°	0.5545	0.5560	0.5575	0.5595	0.5610	0.5625	0.5640	0.5655	0.5670	0.5685	0.5700	23°
24°	0.5520	0.5535	0.5550	0.5565	0.5580	0.5595	0.5610	0.5625	0.5640	0.5655	0.5670	24°
25°	0.5490	0.5505	0.5520	0.5535	0.5550	0.5565	0.5580	0.5595	0.5610	0.5625	0.5640	25°
26°	0.5460	0.5475	0.5490	0.5505	0.5520	0.5535	0.5550	0.5565	0.5580	0.5595	0.5610	26°
27°	0.5430	0.5445	0.5460	0.5475	0.5490	0.5505	0.5520	0.5535	0.5550	0.5565	0.5580	27°
28°	0.5400	0.5415	0.5430	0.5445	0.5460	0.5475	0.5490	0.5505	0.5520	0.5535	0.5550	28°
29°	0.5370	0.5385	0.5400	0.5415	0.5430	0.5445	0.5460	0.5475	0.5490	0.5505	0.5520	29°
30°	0.5340	0.5355	0.5370	0.5385	0.5400	0.5415	0.5430	0.5445	0.5460	0.5475	0.5490	30°
t	752	754	756	758	760	762	764	766	768	770	772	*t*

perature of the solutions below 20°. In the third stage when the nitric oxide is being absorbed by the permanganate, the Hempel pipette should be shaken not faster than twice per second. This is to prevent the breaking off of small gas bubbles.

It is especially necessary that in the first stage the removal of air be complete. This is assured by shaking the solution in the deaminizing bulb back each time, in this stage, until the bulb is two-thirds filled with nitric oxide.

SUGAR

Benedict Modification of the Method of Lewis and Benedict[1]

Principle.—The red color obtained by heating a glucose solution with picric acid and sodium carbonate is employed as the basis of the colorimetric determination. The blood protein is removed by precipitation with picric acid.

Procedure.—Two c.c. of blood are aspirated through an hypodermic needle and a piece of rubber tubing into an Ostwald pipette, a little powdered potassium oxalate in the tip of the pipette preventing clotting. (It may be more convenient to draw about 5 c.c. of blood directly into a test-tube containing a little powdered potassium oxalate and removing 2 c.c. portions of this with the Ostwald pipette.) The blood is drawn up a little above the mark and the end of the pipette is closed with the finger. After the rubber tubing and needle are disconnected, the blood is allowed to flow back to the mark and is discharged at once into a 25 c.c. volumetric flask, or into a large test-tube graduated at 12.5 c.c. and at 25 c.c. The pipette is twice rinsed with distilled water, these washings being added to the blood. The contents of the flask are shaken to insure thorough mixing and a consequent laking or hemolysis of the blood, which is practically complete after a minute or two. A solution of sodium picrate and picric acid is added to the 25 c.c. mark (using a few drops of alcohol to dispel foam if necessary) and the mixture thoroughly shaken. After a minute or two (or longer) the mixture is poured upon a dry filter, and the clear filtrate collected in a dry beaker. Exactly 8 c.c. of the filtrate are measured into a large test-tube bearing

[1] Benedict: Jour. Biol. Chem., 1918, 34, 203. Lewis and Benedict: Jour. Biol. Chem., 1915, 20, 61.

graduations at the 12.5 c.c. and 25 c.c. mark, and 1 c.c. of 20 per cent (anhydrous) sodium carbonate solution is added. The tube is plugged with cotton and immersed in boiling water for ten minutes. (Longer heating up to half an hour makes no change in the color.) It is then removed, and the contents are cooled under running water and diluted to 12.5 c.c. or to 25 c.c. depending on the depth of color. (Occasionally the final filtrates in this or other picric acid methods develop a little turbidity during heating. Unless such turbidity is fairly marked it is of no account. When desired, the final colored solution may be filtered through a small folded filter into the colorimeter cup.) At any time within a half an hour the colored solution is compared in a colorimeter with a suitable standard solution, the standard being set at a height of 15 mm.

The standard solution may be simultaneously prepared from pure glucose by treating 0.64 mg. of glucose in 4 c.c. of water with 4 c.c. of the picrate-picric acid solution and 1 c.c. of the carbonate, and heating for ten minutes in boiling water and then diluting to 12.5 c.c. A permanent standard solution may be prepared from picramic acid or from potassium dichromate as mentioned below. The potassium dichromate standard does not match the unknown with absolute exactness, but can be employed with satisfactory results when pure picramic acid is not obtainable.

Calculation.—If directions are followed exactly the calculation is as follows:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \div 10 = \text{per cent of sugar in the original blood.}$$

Where the final dilution of the unknown is made to 25 c.c. instead of 12.5 c.c. the final figure is, of course, multiplied by two.

Preparation of Solutions. *Picrate-picric Acid Solution.*—To prepare the picrate-picric acid solution, place 36 gms. of dry powdered picric acid in a liter flask or stoppered cylinder, add 500 c.c. of 1 per cent sodium hydroxide solution, and 400 c.c. of hot water. Shake occasionally until dissolved. Cool and dilute to 1 liter.

Permanent Standard.—The picramic acid standard is best prepared from a stock solution containing 100 mg. of picramic acid and 200 mg. of sodium carbonate per liter. One hundred

twenty-six c.c. of this solution are treated with 1 c.c. of the 20 per cent carbonate solution and 15 c.c. of the picrate-picric acid solution, and diluted to 300 c.c. with distilled water. This solution matches exactly the color obtained by treating 0.64 mg. of glucose, as in the above method and diluting to 12.5 c.c.

The standard prepared from potassium dichromate contains 800 mg. of pure potassium dichromate in a liter of water.

RELATIVE HYDROGEN ION CONCENTRATION OF THE BLOOD

Method of Levy, Rowntree, and Marriott [1]

Principle.—The blood is dialyzed against normal salt solution and the H ion concentration of the protein-free dialyzate is determined by the indicator method, using phenolsulphonephthalein.

Procedure.—One to 3 c.c. of clear serum or of blood is run, by means of a blunt-pointed pipette, into a dialyzing sac which has been washed outside and inside with salt solution. The sac is lowered into a small test-tube (100 by 10 mm., inside measurements), containing 3 c.c. of salt solution, until the fluid on the outside of the sac is as high as on the inside. From five to ten minutes are allowed for dialysis. The collodion sac is removed and 5 drops of the indicator (0.01 per cent solution of phenolsulphonephthalein) are thoroughly mixed with the dialyzate. The tube is then compared with the standards until the corresponding color is found, which indicates the hydrogen ion concentration present in the dialyzate. Readings should be made immediately against a white background. Results are expressed in logarithmic notation.

Oxalated blood from normal individuals gives a dialyzate with a P_H varying from 7.4 to 7.6, while that of serum ranges from 7.6 to 7.8. In clinical acidosis figures from 7.55 to 7.2 have been noted by this method for serum and for oxalated blood from 7.3 to 7.1. A rise in the H ion concentration of the blood is significant because it indicates a failure on the part of the protective mechanism of the body to preserve the proper reaction.

Preparation of Sacs.—One ounce of celloidin is dissolved in 500 c.c. of a mixture of equal quantities of ether and ethyl alcohol. The solid swells up and dissolves with occasional gentle shakings,

[1] Levy, Rowntree and Marriott: Arch. Int. Med., 1915, 16, 389.

in forty-eight hours. As a small amount of brown sediment separates out at first, the solution should stand for at least three or four days, after which the clear supernatant solution is ready for use. A small test-tube (120 by 9 mm., inside measurement) is filled with this mixture, inverted, and half the contents poured out. The tube is then righted, and the collodion allowed to fill the lower half again. A second time it is inverted and rotated on its axis, the collodion being drained off. Care must be taken to rotate the tube, in order to secure a uniform thickness throughout. The tube is clamped in the inverted position and allowed to stand for ten minutes, until the odor of ether finally disappears. It is filled five or six times with cold water, or it is allowed to soak five minutes in cold water. A knife blade is run around the upper rim, so as to loosen the sac from the rim of the test-tube, and a few cubic centimeters of water are run down between the sac and the glass tube. By gentle pulling the tube is extracted, after which it is preserved by complete immersion in water.

The Salt Solution.—The blood or serum is dialyzed against an 0.8 per cent sodium chloride solution.

Before applying the test, it is necessary to ascertain that the solution is free from acids other than carbonic. To determine this, a few cubic centimeters of the salt solution are placed in a Jena test-tube and 1 or 2 drops of the indicator added, whereupon a yellow color appears. On boiling carbon dioxide is expelled, and the solution loses its lemon color and takes on a slightly brownish tint. In the absence of this change other acids are present, and the salt solution is therefore not suitable. If, on the other hand, on adding the indicator pink at once appears, the solution is alkaline and hence cannot be used.

Preparation of Standard Colors.—Standard phosphate mixtures are prepared according to Sörensen's directions as follows:

1/15 mol. acid or primary potassium phosphate. 9.078 gms. of the pure recrystallized salt (KH_2PO_4) is dissolved in freshly distilled water and made up to 1 liter.

1/15 mol. alkaline or secondary sodium phosphate. The pure recrystallized salt ($Na_2HPO_4 \cdot 12H_2O$) is exposed to the air for from ten days to two weeks, protected from dust. Ten molecules of water of crystallization are given off and a salt of the formula $Na_2HPO_4 \cdot 2H_2O$ is obtained. 11.876 gms. of this is dissolved in freshly distilled water and made up to 1 liter. The solution

should give a deep rose-red color with phenolphthalein. If only a faint pink color is obtained, the salt is not sufficiently pure.

The solutions are mixed in the proportions indicated below to obtain the desired P_H.

TABLE FOR PREPARATION OF STANDARD COLORS

P_H	6.4	6.6	6.8	7.0	7.1	7.2	7.3	7.4
Primary potassium phosphate c.c.	73	63	51	37	32	27	23	19
Secondary sodium phosphate c.c.	27	37	49	63	68	73	77	81

P_H	7.5	7.6	7.7	7.8	8.0	8.2	8.4
Primary potassium phosphate c.c.	15.8	13.2	11.0	8.8	5.6	3.2	2.0
Secondary sodium phosphate c.c.	84.2	86.8	89.0	91.2	94.4	96.8	98.0

THE HALDANE GAS-ANALYSIS APPARATUS [1]

Principle.—The Haldane method of analysis of expired air is simple and easily learned. The apparatus (Fig. 4) consists of a gas burette, a control burette of the same size (both surrounded with a water jacket), and bulbs containing dilute caustic potash or soda solution for the absorption of the carbon dioxide and an alkaline pyrogallate solution for the absorption of the oxygen. The gas burette is connected with the bulbs by a two-way stopcock, which allows a sample of gas to pass into either bulb. A control tube (10) is put into connection with the burette through a manometer tube, which is connected with the alkali bulb, and can be made to compensate for any changes in temperature that may occur during the course of the analysis. For an analysis the gas is transferred to the burette from the sampling tube, saturated with water vapor over mercury, and then measured, after which

[1] Macleod: Physiology and Biochemistry in Modern Medicine, 2d Edition, p. 559.

it is transferred into the caustic solution to free it from CO_2, and returned to the burette to determine the loss of volume due to CO_2 absorption. It is then transferred into the alkaline pyrogallate solution, which frees it from oxygen, after which it is again brought back to the burette to determine the loss in volume due to the absorption of the oxygen.

The Apparatus.—The detail of the Haldane apparatus is shown in the accompanying cut. The measuring burette **1** holds 21 c.c. The bulb is of 15 c.c. capacity, and the graduated stem, which is about 4 mm. in bore and 60 cm. in length, is graduated to 0.01 c.c. from 15 c.c. to 21 c.c. The stopcock at the top of the burette is double-bored, so that in one position air can be drawn in from a gas sampler **2** and in another sent into the absorption bulbs **3.** The lower part of the burette extends through the rubber cork at the bottom of the water jacket **4.** A piece of rubber tubing is attached to the bottom of the burette and is passed through a metal tube, furnished on its inside with a metal disk which presses against the rubber tubing, the pressure being controlled by means of a fine adjusting screw **6.** Below this a glass stopcock **7** connects with rubber tubing to the mercury leveling bulb **5.** The absorption bulb for CO_2, containing 20 per cent NaOH or KOH **9,** is put in connection with the burette by suitably turning

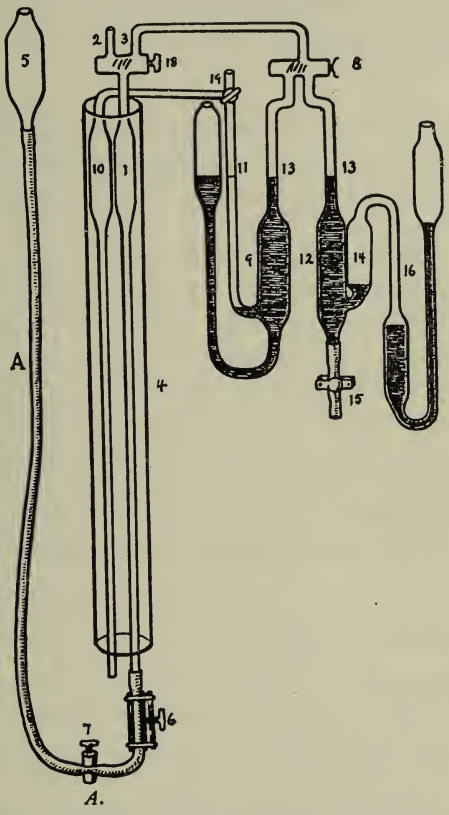

FIG. 4.—Haldane Gas Apparatus. (Macleod.)

stopcocks **3** and **8.** (The stopcock **8** is double-bored, so that the tube leading from the burette can be brought into connection with either **9** or **12.**) The control burette **10** is also in connection with this bulb through the manometer tube **11.** (This tube also has a three-way stopcock **19,** so that it may be opened to the outside.) Any variation in temperature which may occur during the analysis will cause the level of the alkaline solution in the manometer to change.

When final readings of the shrinkage of volume are made, the level of the caustic solution is returned to the level of that in the manometer. By so doing any error due to temperature changes is avoided, since change in temperature must be equal in the two burettes.

The absorption bulb **12** for oxygen is filled with a solution made by dissolving 10 gms. of pyrogallic acid in 100 c.c. of a nearly saturated KOH solution. The specific gravity of the KOH should be 1.55, which is obtained approximately by dissolving the sticks (pure by alcohol) in an equal weight of water. The mark **13** on the stem of the bulb indicates the level at which the solution should stand. Enough pyrogallate solution is introduced through tube **15** to fill bulbs **12** and **14** two-thirds full. Then pyrogallate solution is poured into tube **16** until the difference in level of the fluids is sufficient to produce enough pressure to raise the level of the pyrogallate solution in **12** to the level **13** on the stem. Stopcock **8** must be open during this procedure. It may be necessary to add or take away a little pyrogallate solution through **15** to attain the above level.

Care must be taken to allow for complete absorption of oxygen from the air that is entrapped between **14** and **16** before an analysis is made; otherwise changes will be produced in the level of the pyrogallate solution. The air in the capillary tubing connecting the burettes with the absorption bulbs must also be freed of CO_2 and O_2. This can be accomplished by making a dummy analysis of atmospheric air before the real analysis. Great care must be taken to have atmospheric pressure in all the tubes at the start of the analysis. This is accomplished by opening the stopcock in the burette first to atmospheric air and then to the absorption bulbs, until no further change in the level of the fluids in the stems of the absorption bulbs occurs. This level is then marked and used as the standard. A small amount of water in the burette over

the mercury assures saturation of the air with water vapor. Time for drainage must be allowed before making readings.

A very serviceable *sampling tube* for the transfer of air can be made from a 30 c.c. ground-glass syringe, to which is attached a two-way stopcock. The dead space in these syringes is washed out by working the piston back and forth several times. A thin coating of vaseline prevents leakage of the gas. We have found that these sampling tubes will retain a sample of expired air without change up to eight hours.

Manipulation of Apparatus.—The sampling syringe **20** is attached to opening **2** of the burette, and its stopcock **17** opened to atmospheric air. The level of the mercury is raised to the level of the stopcock of the syringe and is then turned so that syringe and burette are in communication. The bulb of mercury is lowered so that the mercury falls in the burette. This draws the piston of the syringe with it, and fills the burette with air from the syringe. It is advisable to put a little positive pressure on the piston of the syringe in the maneuver to prevent possible leakage. When all of the air is in the burette a slight positive pressure is produced in the burette by gently pressing on the piston, and immediately thereafter the stopcock on the syringe **17** is again turned to the original position. This allows the pressure of air in the burette to come to that of the atmosphere. The height of the mercury is now adjusted to a convenient height in the burette by closing cock **7** and turning the milled screw **6**. The cock **8** is now made to communicate with the absorption bulbs. If the air in the burette is at atmospheric pressure, no change will occur in the level of the fluids. The reading is then taken on the burette.

The next step in the analysis consists in turning stopcock **8** to communicate with the caustic soda solution in bulb **9**, and the leveling tube **5** is raised, forcing mercury into the burette and the air into bulb **9**. The gas is passed back and forth several times until absorption is complete, as can be determined by the fact that the level of the mercury in the burette remains constant when the fluid in the bulb is returned to its original level **13** on the stem. In this adjustment it is convenient to make the gross leveling by the mercury bulb and the fine leveling by closing **7** and turning **6** until the fluid in **9** is at the original height. The reading on the burette indicates the loss in volume due to the CO_2 absorbed.

The oxygen is removed by a similar procedure, the gas being passed into the alkaline pyrogallate solution by turning cock **8** to communicate with bulb **12.** The absorption of oxygen is slower than for CO_2, and more care must be taken to get complete absorption. The air in the tubing between the fluid in **9** and stopcock **8** must be washed out several times in order to get the oxygen which is left in it after the absorption of the CO_2. When this is complete, the final reading on the burette is made and the loss in volume from the second reading represents the oxygen.

The Calculations.—The calculation of the percentile composition of the air is represented in the following example of an actual analysis:

(The temperature and barometric pressure as taken at the time of the experiment were 20° C. and 747 mm. Hg.)

CO_2 *Analysis*—

1st reading of burette	20.00
2d reading of burette after absorption of CO_2	19.20
CO_2 absorbed	0.80

$0.80 \div 20 = 4.0$ per cent CO_2 in expired air.

O_2 *Analysis*—

2d reading of burette	19.20
3d reading of burette after absorption of CO_2	15.90
O_2 absorbed	3.30

$3.30 \div 20 = 16.50$ per cent of O_2 in expired air.

TABLE FOR REDUCING GASEOUS VOLUMES TO NORMAL TEMPERATURE AND PRESSURE

The observed volume, when multiplied by the factor corresponding to the temperature and pressure, will give the volume of the expired air reduced to 0° C. and 760 mm.

mm.	15°	16°	17°	18°	19°	20°	21°	22°	23°	24°	25°
720	0.898	0.894	0.891	0.888	0.885	0.882	0.880	0.877	0.873	0.870	0.867
730	0.910	0.907	0.904	0.901	0.897	0.894	0.891	0.888	0.885	0.882	0.879
740	0.922	0.919	0.916	0.913	0.910	0.907	0.904	0.901	0.897	0.894	0.891
750	0.935	0.932	0.928	0.925	0.922	0.919	0.916	0.913	0.910	0.907	0.904
760	0.947	0.944	0.941	0.938	0.934	0.931	0.928	0.925	0.922	0.919	0.916
770	0.960	0.957	0.953	0.950	0.948	0.945	0.940	0.936	0.933	0.930	0.927

BLOOD GAS ANALYSIS

Method of Henderson and Smith [1]

Principle.—Modification of the method of Barcroft and Haldane. The apparatus used consists of the Haldane gas analyzer in either its original or modified form and a special small graduated diffusion tube.

Solutions Employed.—(1) *Dilute ammonia* (1 c.c. of concentrated ammonia in 500 c.c. of distilled water) kept tightly stoppered to avoid absorption of CO_2 from the air (or freed from carbonate with barium hydroxide and ammonium sulphate). A little saponin is dissolved in the ammonia solution when an oxalate is used to prevent the blood sample from clotting. (2) *A 10 per cent solution of potassium ferricyanide.* (3) *A 20 per cent solution of tartaric acid.*

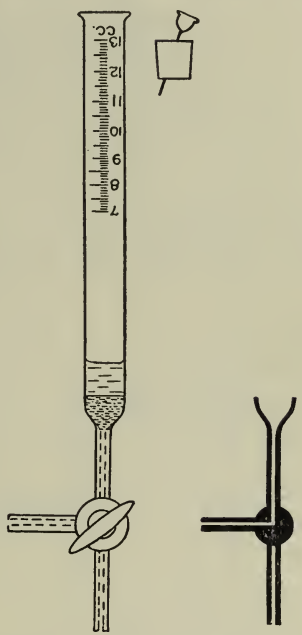

Fig. 5.—The Blood Gas Diffusion Tube. The plug of the stop-cock is bored only in a right angle, not in a T, so that only two of the openings are connected at once. The open end of the tube is graduated as shown. This end of the tube is closed with a rubber stopper cut to about a half or a third the usual length, so that the needle of a small all glass hypodermic syringe can be thrust through it. (Jour. Biol. Chem., 1918, 33, 40.)

OXYGEN ANALYSIS

Procedure.—The stop-cock of the diffusion tube is closed (i.e., turned to connect the side and end nipples); 1.5 c.c. of the ammonia solution are placed in the tube; and 1 c.c. of the blood to be analyzed is introduced below the ammonia by means of a pipette. A short close-fitting rubber stopper is inserted in the large end. An hypodermic needle (disconnected from its syringe) is passed through this

[1] Henderson and Smith: Jour. Biol. Chem., 1918, 33, 39.

stopper to allow the escape of the air compressed by the insertion of the stopper. The needle is withdrawn, and the

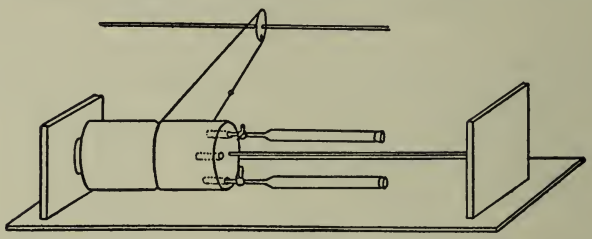

Fig. 6.—Rotating Apparatus. (Jour. Biol. Chem., 1918, 33, 41.)

tube is rotated gently to mix the blood and ammonia until the corpuscles are completely laked. A small, all-glass syringe fitted with an hypodermic needle is then filled with the ferricyanide solution and the air is expelled. The needle is then thrust through the rubber stopper until the point projects in the interior of the tube, and 0.25 c.c. of the ferricyanide solution are injected. The needle is then carefully withdrawn.

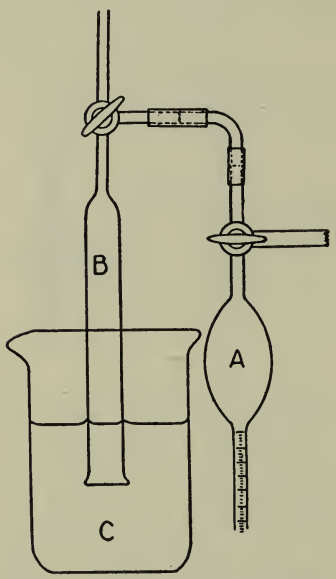

Fig. 7.—*A*, Gas Burette of Analyzer; *B*, Diffusion Tube; *C*, Beaker of Water. (Jour. Biol. Chem., 1918, 33, 43.)

The tube is now placed for five minutes in a rotating apparatus (Fig. 6) consisting of a block of wood turning on an iron rod and belted to a slowly moving motor or shafting. There are a number of holes bored in the sides of the block of wood into which diffusion tubes can be inserted. The rate of revolution should be not more than 40 or 50 times a minute. During the rotation the contents of the tube spread in a thin film along the walls and allow complete diffusion of the liberated oxygen into the air of the tube.

The tube is next connected glass to glass with the gas analyzer by a short piece of heavy rubber tubing in the manner shown in

Fig. 7. The mercury bulb of the analyzer is lifted until mercury runs out through the top of the analyzer and fills the capillary tube of the blood gas diffusion tube. The lower end of the diffusion tube is placed in a beaker of water and the stopper is withdrawn under water. The beaker is raised or lowered until the levels of the fluid inside and outside the tube are the same, and the volume of the air in the tube is read. The stop-cock at the top of the diffusion tube is then turned and a sample of the air within the tube is drawn over into the analyzer and analyzed for oxygen.

The residual gas from this analysis is nitrogen. As the percentages of oxygen and nitrogen in atmospheric air are known, it is easy to calculate from the volume of this residual nitrogen the exact amount of oxygen which would have been found by the analysis if no oxygen had been given off by the blood. To do this multiply the residual nitrogen by $\frac{20.93}{79.04}$, or 0.265. The volume of oxygen thus calculated to have been in the air is subtracted from that found. A correction is then made for the volume of gas which remained in the diffusion tube and connections after the sample was drawn. Corrections are also made for barometric pressure and temperature, for the volume of gas is always expressed as it would be at 0° C. and 760 mm. barometer. The following is an example of this calculation:

Volume of air in diffusion tube ..... = 10.1 c.c. (1)
Volume taken for analysis ..... = 9.440 c.c. (2)
Nitrogen remaining after absorption of oxygen ..... = 7.342 c.c. (3)
Oxygen absorbed, i.e., (2) − (3) ..... = 2.098 c.c. (4)
Volume of atmospheric oxygen in volume of gas taken for analysis, i.e., $(3) \times \frac{20.93}{79.04}$ ..... = 1.945 c.c. (5)
Oxygen from blood in volume of gas analyzed, i.e., (4) − (5) ..... = 0.153 c.c. (6)
Total oxygen from blood, i.e., $(6) \times \frac{(1)}{(2)}$ ..... = 0.163 c.c. (7)
Oxygen reduced from barometric pressure 755 and temperature 20° to 760 and 0°, i.e.,

$$(7) \times \frac{760}{755} \times \frac{273}{(273+20)} * = 0.147 \text{ c.c.} \quad (8)$$

Volumes per cent oxygen in blood, i.e., (8) × 100 ..... = 14.7

* Tables for these factors of pressure and temperature will be found in any handbook of chemical constants.

Comparative determinations of the oxygen content of the blood by the Barcroft-Haldane method using the Brodie apparatus (5) and by this method gave the following results:

Barcroft-Haldane Method	*Gas Analysis Method*
16.2	16.3
16.5	16.1

The two methods are of approximately the same order of accuracy. To one accustomed to the use of the gas analyzer, but not in practice for the use of the Barcroft-Haldane method, in its usual form, this modification is believed to be decidedly the easier.

The precision of the method may be increased,—at least theoretically—by filling the diffusion tube initially with nitrogen (from the analyzer) instead of air, inserting the stopper under water, and injecting the hypodermic syringe. But this refinement takes time and requires extreme care to avoid admitting the least trace of air.

CO_2 DETERMINATION

The CO_2 content of the blood is determined in exactly the same manner and with the same tube, merely using the tartaric acid solution instead of ferricyanide. (When the amount of gas in the blood is very high it is advisable to use only half quantities of blood and reagent; namely, 0.75 c.c. of blood, and 0.10 c.c. of tartaric acid.) Owing to the solubility of CO_2 it is necessary in the calculation of the results to take into account that part of the gas which remains in solution in the acidified, diluted blood. This correction for any temperature may be obtained from Curve 1 in Fig. 8.

A typical calculation of the results obtained is as follows:

Volume of air at 18° C. (compressed) in diffusion tube before removing stopper	= 10.9	c.c.	(1)
Volume of air (at atmospheric pressure) in diffusion tube after removing stopper	= 11.1	c.c.	(2)
Volume taken for analysis	= 7.774	c.c.	(3)
Gas remaining after absorption of CO_2	= 7.506	c.c.	(4)
CO_2 absorbed, i.e., (3) − (4)	= 0.268	c.c.	(5)
Total CO_2 in air in tube, i.e., (5) $\times \frac{(2)}{(3)}$	= 0.383	c.c.	(6)

CO_2 in solution in acidified blood, i.e., volume of liquid (2.75 c.c.) $\times$ solubility coefficient (1.0) $\times CO_2$ in air in tube $\frac{(6)}{(1)}$ ..... = 0.096 c.c. (7)

Total CO_2 from blood, i.e., (6) + (7) ..... = 0.479 c.c. (8)

CO_2 reduced from barometric pressure 762 and temperature 26° to 760 and 0°, i.e.,

$$(8) \times \frac{760}{762} \times \frac{273}{(273+18)} = 0.448 \text{ c.c.} \quad (9)$$

Volumes per cent CO_2 in blood, i.e., (9) $\times$ 100 ..... = 44.8

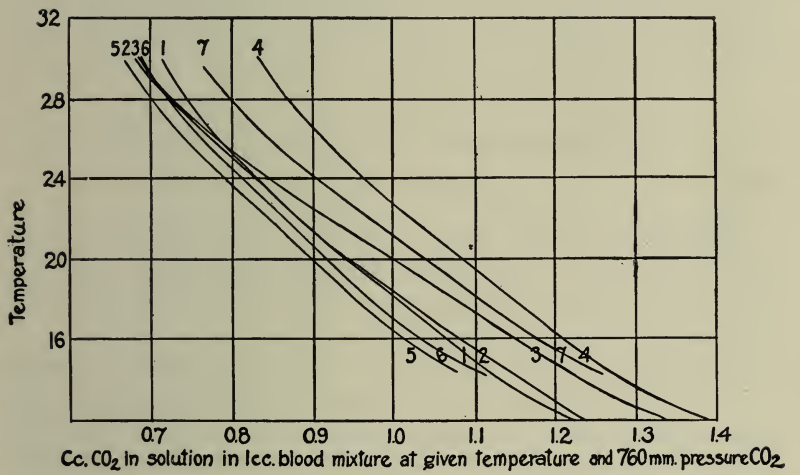

FIG. 8.—Curves Expressing Solubility of CO_2. The acid-blood mixture of Curves 1, 2, 3, 4, and 7 was made as follows: ammonia 1.5 c.c., blood 1.0 c.c., tartaric acid 0.25 c.c. The mixture of Curves 5 and 6 was made as follows: ammonia 2.0 c.c., blood 1.0 c.c., tartaric acid 0.5 c.c. Curves 1 and 2, fresh human blood; Curve 3, fresh cat blood; Curve 4, blood of dog anesthetized with chloratone and alcohol; Curve 5, fresh pig blood; Curves 6 and 7, human serum.

Comparison of the results obtained with the original Barcroft-Haldane method and with this modification shows in every case that the latter gives figures 2 to 3 volumes per cent higher than those obtained with the older method. This is due to the fact that the heavy precipitate by adding both ferricyanide and acid to blood renders it extremely difficult in the Brodie apparatus to shake the last trace of CO_2 out of the thick coagulum.

Simultaneous Determination of Oxygen and CO_2

There appears no reason (except that just mentioned) why both gases should not be determined on a single sample of blood by first liberating the oxygen and then the CO_2. This can in fact be done (using half quantities of blood and reagents or in diffusion tubes of 20 c.c. capacity) if a small glass rod or some lead shot is put into the tube to assist in breaking up the heavy masses of the precipitate formed by the addition of the acid after the ferricyanide. Practically, however, unless the quantity of blood available is extremely limited it is easier and more accurate to make the two determinations separately. Duplicate determinations for both gases independently can be made by this method in half an hour.

OXYGEN-BINDING CAPACITY OF BLOOD

Method of Van Slyke[1]

Principle.—Blood is laked and oxygen combined with hemoglobin is set free. The oxygen is then collected and measured. From this volume of gas the oxygen bound by the hemoglobin is calculated.

Procedure.—Five to 10 c.c. of blood is introduced into a separatory funnel or bottle and distributed in a thin layer about the inner wall, so that maximum contact with the air is assured. The vessel is rotated for three or four minutes so that the blood is kept in a thin layer, or it may be shaken fifteen or more minutes on a mechanical shaker. The blood is then transferred to a cylinder or heavy-walled tube. The blood gas apparatus is now prepared by introducing into it 5 drops of redistilled caprylic alcohol and 6 c.c. of ammonia solution made by diluting 4 c.c. of concentrated ammonia to a liter. If saponin is available the diluted ammonia should be made to contain about 1 mg. per cubic centimeter. The apparatus is evacuated and the air extracted from the ammonia by shaking for about fifteen seconds. The extracted air is expelled and the process repeated to make sure that no air is left in the solution. Just before the blood is introduced about 2 c.c. of the air-free ammonia is forced up into the cup of the apparatus. The aerated blood is now thoroughly stirred with a rod to assure even distribution of the corpuscles, and 2 c.c. is drawn into a pipette

[1] Medical War Manual; No. 6, 1918, p. 129.

and run under the ammonia in the cup of the apparatus. All but a few drops of the liquid in the cup is now run into the 50 c.c. chamber, the ammonia following the blood and washing it in. A few additional drops of the ammonia may be added from a dropper to make the washing complete.

The blood and ammonia in the apparatus are mixed and allowed to stand until the blood is *completely laked.* This requires about thirty seconds when saponin is present and five minutes when it is not. After laking is complete 0.4 c.c. of a saturated (40 gms. to 100 c.c. of water) potassium ferricyanide solution is introduced to set free the oxygen combined with the hemoglobin. (The cyanide solution is made air-free by boiling or by shaking in an evacuated flask and is kept in a burette under a layer of paraffin oil 2 or 3 cm. thick to exclude air.) The apparatus is now evacuated until only a few drops of mercury remain above the lower stop-cock, and is shaken, preferably with a rotary motion, to whirl the blood in a thin layer about the wall of the chamber. If the blood was completely laked before the cyanide was added, extraction of the oxygen is completed in half a minute. The water solution is now drawn down into the bulb of the apparatus below the lower cock, and the extracted gases measured as in the determination of carbon dioxide. After the gas volume has been read the chamber is evacuated, and the blood readmitted and shaken again for thirty seconds in the vacuum. The reading is then repeated. If it shows an increase a third extraction should be performed.

In order to determine the oxygen bound by the hemoglobin it is necessary to subtract from the gas measured the volume of air physically dissolved by 2 c.c. of blood at atmospheric pressure and the prevailing room temperature. The volume of gas thus corrected may be reduced to standard conditions, 0°, 760 mm., by multiplying by $(0.999.-0.0046t)\times\frac{\text{barometer}}{760}$, t being the temperature in degrees Centigrade. If this result is multiplied by 50 it gives the cubic centimeters of oxygen bound by the hemoglobin in 100 c.c. of blood. The amounts of air dissolved are given in the table on page 126, which also gives the factors by which one may directly transpose the readings into terms of percentage of normal hemoglobin on the basis of Haldane's average, viz., 18.5 per cent oxygen = 100 per cent hemoglobin.

FACTORS FOR CALCULATING HEMOGLOBIN FROM OXYGEN BOUND BY 2 c.c. OF BLOOD

Temperature	Air dissolved by 2 c.c. of blood. Subtract from gas volume read in apparatus in order to obtain *corrected gas volume*, representing O_2, set free from hemoglobin.	Factor by which Corrected Gas Volume is Multiplied in Order to Give:	
		Oxygen chemically bound by 100 c.c. of blood.	Per cent hemoglobin calculated on the basis: 18.5 per cent oxygen = 100 per cent hemoglobin.
C°.	c.c.	c.c.	Per Cent
15	0.037	$46.5\times\frac{B}{760}$	$251\times\frac{B}{760}$
16	0.036	$46.3\times\frac{B}{760}$	$250\times\frac{B}{760}$
17	0.036	$46.0\times\frac{B}{760}$	$249\times\frac{B}{760}$
18	0.035	$45.8\times\frac{B}{760}$	$247\times\frac{B}{760}$
19	0.035	$45.6\times\frac{B}{760}$	$246\times\frac{B}{760}$
20	0.034	$45.4\times\frac{B}{760}$	$245\times\frac{B}{760}$
21	0.033	$45.1\times\frac{B}{760}$	$244\times\frac{B}{760}$
22	0.033	$44.9\times\frac{B}{760}$	$242\times\frac{B}{760}$
23	0.032	$44.7\times\frac{B}{760}$	$241\times\frac{B}{760}$
24	0.032	$44.4\times\frac{B}{760}$	$240\times\frac{B}{760}$
25	0.031	$44.2\times\frac{B}{760}$	$239\times\frac{B}{760}$
26	0.030	$44.0\times\frac{B}{760}$	$237\times\frac{B}{760}$
27	0.030	$43.7\times\frac{B}{760}$	$236\times\frac{B}{760}$
28	0.029	$43.5\times\frac{B}{760}$	$235\times\frac{B}{760}$
29	0.029	$43.3\times\frac{B}{760}$	$234\times\frac{B}{760}$
30	0.028	$43.1\times\frac{B}{760}$	$233\times\frac{B}{760}$

It is advisable after one 2 c.c. portion of a blood sample has been analyzed to aerate the remainder of the sample a second time and repeat the determination in order to make certain that the first sample was completely saturated with oxygen.

Example—

Observed gas volume = 0.45 c.c. at 20°, 760 mm.
Correction for dissolved air = 0.034.
Corrected gas volume = 0.416 c.c.
Hemoglobin = $0.416 \times 245 = 102$ per cent.

ALKALI RESERVE

Direct Method. Carbon Dioxide Capacity of the Plasma (Van Slyke and Cullen) [1]

Principle. The plasma from oxalated blood is shaken in a separatory funnel filled with an air mixture whose carbon dioxide tension approximates that of normal arterial blood, by which treatment it combines with as much carbon dioxide as it is able to hold under normal tension. A known quantity of the saturated plasma is then acidified within a suitable pipette, and its carbon dioxide is liberated by the production of a partial vacuum. The liberated carbon dioxide is then placed under atmospheric pressure, its volume carefully measured, and the volume corresponding to 100 c.c. of plasma calculated.

Apparatus.—The apparatus used in the estimation of the carbon dioxide content of the plasma is illustrated in Fig. 9. It is made of strong glass in order to stand the weight of mercury without danger of breaking, and is held in a strong screw clamp the jaws of which are lined with thick pads of rubber. In order to prevent accidental slipping of the apparatus from the clamp, an iron rod of 6 or 8 mm. diameter should be so arranged as to project under cock *f* between *c* and *d*.

Three hooks or rings at the levels 1, 2, and 3 serve to hold the leveling bulb at different stages of the analysis. The bulb is connected with the bottom of the apparatus by a heavy walled rubber tube.

[1] Van Slyke and Cullen: Jour. Biol. Chem., 1917, 30, 289. Van Slyke: Jour. Biol. Chem., 1917, 30, 347.

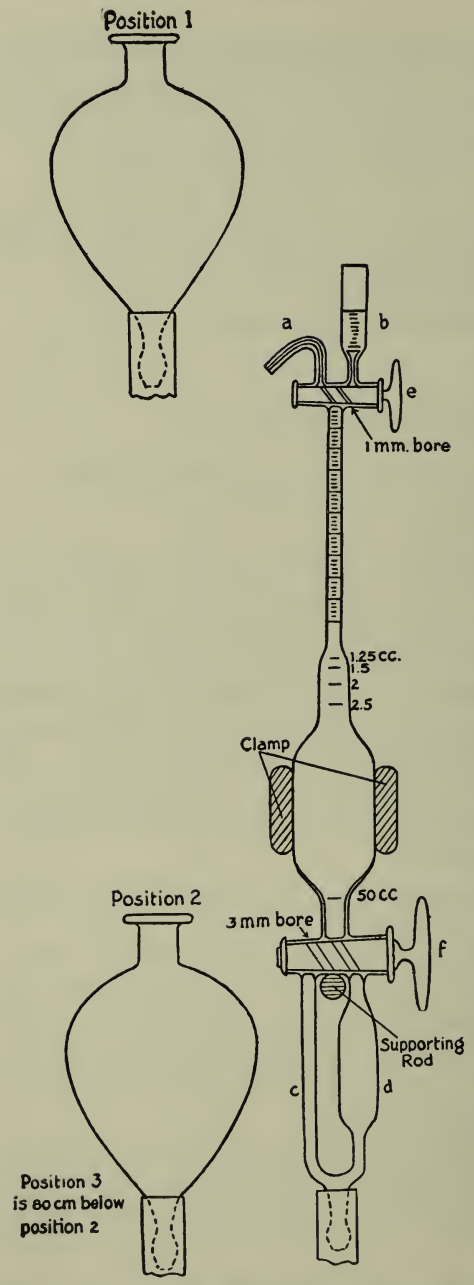

Fig. 9.—Van Slyke Carbon Dioxide Apparatus. (Jour. Biol. Chem., 1917, 30, 289; 1917, 30, 347.) (Hawk.)

It is necessary, of course, that both stop cocks should be properly greased and air tight, and it is also essential that they (especially *f*) shall be held in place so that they cannot be forced out by pressure of the mercury. Rubber bands may be used for this purpose but it is suggested that elastic cords of fine wire spirals, applied in the same manner as rubber bands, are stronger and more durable.

After a determination has been finished, the leveling bulb is lowered without opening the upper cock, and most of the mercury is withdrawn from the pipette through *c*. The water solution from *d* is readmitted and the leveling bulb being raised to position 1, the water solution, together with a little mercury, is forced out of the apparatus through *a*. (It is well to have a funnel draining into a special vessel to catch the water residues and mercury overflow from *a*. A considerable amount of mer-

cury is thus regained if many analyses are run. It requires only straining through cloth or chamois skin to prepare it for use again.)

Procedure.—*Drawing the Blood.* (For at least an hour before the blood is drawn the subject should avoid vigorous muscular exertion as this, presumably because of the lactic acid formed, lowers the bicarbonate of the blood.) About 6 or 7 c.c. of venous blood are aspirated into a centrifuge tube (see Fig. 10) which contains a little powdered potassium oxalate and some paraffin oil. The tube is subjected to a minimum of agitation after the blood is in it. The slight amount of agitation necessary to assure mixture with the oxalate is accomplished by stirring with the inlet tube, rather than by inverting or shaking. The tube and contents are then centrifuged.

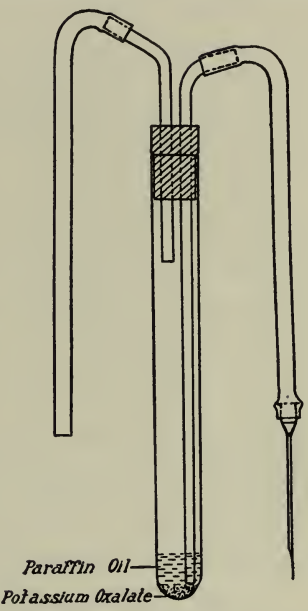

Fig. 10.—Tube Used in Collecting Blood. (Jour. Biol. Chem., 1917, 30, 289.) (Hawk.)

Saturation of Plasma with Carbon Dioxide.—After centrifugation about 3 c.c. of the plasma (if it is desired to keep the plasma

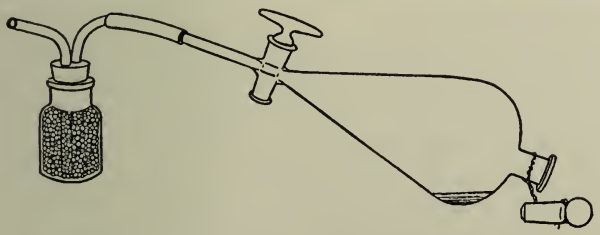

Fig. 11.—Separatory Funnel Used in Saturating Blood Plasma with Carbon Dioxide. (Jour. Biol. Chem., 1917, 30, 289.) (Hawk.)

for the estimation of carbon dioxide at a later time it should be transferred to a paraffined tube, covered with a layer of paraffin oil, stoppered and kept cold; under which conditions it is claimed

that, if sterile, it may be kept for over a week without alteration of its carbon dioxide capacity) are transferred to a 300 c.c. separatory funnel, arranged as in Fig. 11, and the air within the funnel is displaced by either alveolar air from the lungs of the operator or a 5.5. per cent carbon dioxide-air mixture from a tank. In either case the gas mixture must be passed over moist glass beads before it enters the funnel.

When alveolar air is used the operator, without inspiring more deeply than normal, expires as quickly and as completely as possible through the glass beads and separatory funnel. The stopper of the funnel should be inserted just before the expiration is finished, so that there is no opportunity for air to be drawn back into the funnel. In order to saturate the plasma the separatory funnel is turned end over end for two minutes, the plasma being distributed in a thin layer as completely over the surface of the funnel's interior as is possible. After saturation is completed the funnel is placed upright and allowed to stand for a few minutes until the fluid has drained from the walls and gathered in the contracted space at the bottom of the funnel.

Determination of Carbon Dioxide.—A sample of 1 c.c. (or 0.5 c.c. in case the amount of plasma available is very small) accurately pipetted, is allowed to run into the cup *b* in the apparatus represented in Fig. 3, the tip of the pipette remaining below the surface of the plasma as it is added. The cup should have been previously washed out with carbonate-free ammonia, and together with the entire apparatus should have been filled with mercury to the top of the capillary tube by placing the leveling bulb of mercury in position 1.

With the mercury bulb at position 2 and the cock *f* in the position shown in the figure the plasma is admitted from the cup into the 50 c.c. chamber, leaving just enough above the cock *e* to fill the capillary so that no air is introduced when the next solution is added. The cup is washed with two portions of about 0.5 c.c. of water, each portion being added to the pipette in turn. A small drop of caprylic alcohol is then added to the cup and is permitted to flow entirely into the capillary above *e*. Finally 0.5 c.c. of 5 per cent sulphuric acid is run in. (It is desirable to keep the amount of caprylic alcohol small, as larger amounts may appreciably increase results. With plasma 0.02 c.c. is sufficient to prevent foaming and is measured most conveniently from a burette made

by fusing a capillary stop cock to a pipette graduated into 0.01 c.c. divisions.)

It is not necessary that exactly 1 c.c. of wash water and 0.5 c.c. of acid shall be taken, but the total volume of the water solution introduced must extend exactly to the 2.5 c.c. mark on the apparatus, if the table on page 133 is to be used.

If the amount of plasma available is small a little more than 0.5 c.c. are saturated in a 50 c.c. funnel, and exactly 0.5 c.c. used for the estimation of carbon dioxide. In this case the volume of distilled water and acid used to wash the plasma into the apparatus is halved, so that the total volume of water solution introduced is only 1.25 c.c. and in the calculation the observed volume of gas is multiplied by 2.

After the acid has been added a drop of mercury is placed in *b* and allowed to run down the capillary as far as the cock in order to seal the latter. Whatever excess of sulphuric acid remains in the cup is washed out with a little water.

The mercury bulb is now lowered and hung at position 3 and the mercury in the pipette is allowed to run down to the 50 c.c. mark, producing a Torricellian vacuum in the apparatus. When the mercury (not the water) meniscus has fallen to the 50 c.c. mark the lower cock is closed and the pipette is removed from the clamp. Equilibrium of the carbon dioxide between the 2.5 c.c. of water solution and the 47.5 c.c. of free space in the apparatus is obtained by turning the pipette upside down fifteen or more times, thus thoroughly agitating the contents. The pipette is then replaced in the clamp.

By turning the cock *f* the water solution is now allowed to flow from the pipette completely into *d* without, however, allowing any of the gas to follow it. The leveling bulb is then raised in the left hand while with the right the cock is turned so as to connect the pipette with *c*. The mercury flowing in from *c* fills the body of the pipette, and as much of the calibrated stem at the top as is not occupied by the gas extracted from the solution. A few hundredths of a cubic centimeter of water which could not be completely drained into *d* float on top of the mercury in the pipette, but the error caused by reabsorption of carbon dioxide into this small volume of water is negligible if the reading is made at once. The mercury bulb is placed at such a level that the gas in the

pipette is under atmospheric pressure and the volume of the gas is read on the scale.

Calculation.—By means of the table on page 133 the readings on the apparatus can be directly transposed into c.c. of carbon dioxide chemically bound by 100 c.c. of plasma. The barometer reading and room temperature are taken at the time of the determination. For convenience in the calculation values are given below for the ratio $\frac{B}{760}$ over the range usually encountered. (In order to have the column in the pipette exactly balanced by that outside, the surface of the mercury in the leveling bulb should be raised until it is level with the mercury meniscus in the pipette, and then, for entire accuracy, raised above the latter meniscus by a distance equal to 1/14 the height of the column of water above the mercury in the pipette. As the water column is as a rule, only about 10 mm. high, the correction that has to be estimated is less than 1 mm. of mercury, i.e., the entire correction for the water column is not enough to influence results appreciably.

In case the volume of plasma taken for estimation of carbon dioxide content was 0.5 c.c. the observed volume of gas is multiplied by 2 before it is used to calculate the volume per cent of carbon dioxide bound.

Barometer	$\frac{B}{760}$	Barometer	$\frac{B}{760}$
732	0.963	756	0.995
734	0.966	758	0.997
736	0.968	760	1.000
738	0.971	762	1.003
740	0.974	764	1.006
742	0.976	766	1.008
744	0.979	768	1.011
746	0.981	770	1.013
748	0.984	772	1.016
750	0.987	774	1.018
752	0.989	776	1.021
754	0.992	778	1.024

The temperature figures at the heads of columns represent the room temperature at which the samples of plasma are saturated with alveolar carbon dioxide and analyzed. It is assumed that both operations are performed at the same temperature. The figures have been so calculated that, regardless of the room tem-

TABLE FOR CALCULATION OF CARBON DIOXIDE COMBINING POWER OF PLASMA

Observed vol. gas $\times\frac{B}{760}$	c.c. of CO_2 reduced to 0° 760 mm. bound as bicarbonate by 100 c.c. of plasma.				Observed vol. gas $\times\frac{B}{760}$	c.c. of CO_2 reduced to 0° 760 mm. bound as bicarbonate by 100 c.c. of plasma.			
	15°	20°	25°	30°		15°	20°	25°	30°
0.20	9.1	9.9	10.7	11.8	0.60	47.7	48.1	48.5	48.6
1	10.1	10.9	11.7	12.6	1	48.7	49.0	49.4	49.5
2	11.0	11.8	12.6	13.5	2	49.7	50.0	50.4	50.4
3	12.0	12.8	13.6	14.3	3	50.7	51.0	51.3	51.4
4	13.0	13.7	14.5	15.2	4	51.6	51.9	52.2	52.3
5	13.9	14.7	15.5	16.1	5	52.6	52.8	53.2	53.2
6	14.9	15.7	16.4	17.0	6	53.6	53.8	54.1	54.1
7	15.9	16.6	17.4	18.0	7	54.5	54.8	55.1	55.1
8	16.8	17.6	18.3	18.9	8	55.5	55.7	56.0	56.0
9	17.8	18.5	19.2	19.8	9	56.5	56.7	57.0	56.9
0.30	18.8	19.5	20.2	20.8	0.70	57.4	57.6	57.9	57.9
1	19.7	20.4	21.1	21.7	1	58.4	58.6	58.9.	58.8
2	20.7	21.4	22.1	22.6	2	59.4	59.5	59.8	59.7
3	21.7	22.3	23.0	23.5	3	60.3	60.5	60.7	60.6
4	22.6	23.3	24.0	24.5	4	61.3	61.4	61.7	61.6
5	23.6	24.2	24.9	25.4	5	62.3	62.4	62.6	62.5
6	24.6	25.2	25.8	26.3	6	63.2	63.3	63.6	63.4
7	25.5	26.2	26.8	27.3	7	64.2	64.3	64.5	64.3
8	26.5	27.1	27.7	28.2	8	65.2	65.3	65.5	65.3
9	27.5	28.1	28.7	29.1	9	66.1	66.2	66.4	66.2
0.40	28.4	29.0	29.6	30.0	0.80	67.1	67.2	67.3	67.1
1	29.4	30.0	30.5	31.0	1	68.1	68.1	68.3	68.0
2	30.3	30.9	31.5	31.9	2	69.0	69.1	69.2	69.0
3	31.3	31.9	32.4	32.8	3	70.0	70.0	70.2	69.9
4	32.3	32.8	33.4	33.8	4	71.0	71.0	71.1	70.8
5	33.2	33.8	34.3	34.7	5	71.9	72.0	72.1	71.8
6	34.2	34.7	35.3	35.6	6	72.9	72.9	73.0	72.7
7	35.2	35.7	36.2	36.5	7	73.9	73.9	74.0	73.6
8	36.1	36.6	37.2	37.4	8	74.8	74.8	74.9	74.5
9	37.1	37.6	38.1	38.4	9	75.8	75.8	75.8	75.4
0.50	38.1	38.5	39.0	39.3	0.90	76.8	76.7	76.8	76.4
1	39.1	39.5	40.0	40.3	1	77.8	77.7	77.7	77.3
2	40.0	40.4	40.9	41.2	2	78.7	78.8	78.7	78.2
3	41.0	41.4	41.9	42.1	3	79.7	79.6	79.6	79.2
4	42.0	42.4	42.8	43.0	4	80.7	80.5	80.6	80.1
5	42.9	43.3	43.8	43.9	5	81.6	81.5	81.5	81.0
6	43.9	44.3	44.7	44.9	6	82.6	82.5	82.4	82.0
7	44.9	45.3	45.7	45.8	7	83.6	83.4	83.4	82.9
8	45.8	46.2	46.6	46.7	8	84.5	84.4	84.3	83.8
9	46.8	47.1	47.5	47.6	9	85.5	85.3	85.2	84.8
0.60	47.7	48.1	48.5	48.6	1.00	86.5	86.2	86.2	85.7

perature at which saturation and analysis are performed, the table gives the volume (reduced to 0°, 760 mm.) of carbon dioxide that 100 c.c. of plasma are capable of binding when saturated at 20° with carbon dioxide at approximately 41 mm. tension. If the figures in the table are multiplied by 0.94 they give, within 1 or 2 per cent of the carbon dioxide bound at 37°.

TITRATION OF THE BICARBONATE CONTENT OF BLOOD PLASMA

Method of Van Slyke, Stillman and Cullen [1]

Principle.—The bicarbonate content of serum or oxalate plasma is determined by adding an excess of standard acid, removing the carbon dioxide by rotating the solution, and titrating back with standard alkali to the original hydrogen ion concentration of the blood with neutral red as indicator.

Procedure.—In drawing and centrifugating the blood the precautions outlined by Van Slyke and Cullen for preventing loss or accumulation of carbon dioxide and consequent change in the distribution of bicarbonate between corpuscles and plasma, are to be observed. Oxalate plasma is used. Up to the beginning of the analysis, the blood and plasma are handled exactly as described for the carbon dioxide method.

For the analysis, 2 c.c. of plasma are pipetted into a round-bottomed flask of 150 to 200 c.c. capacity, and 5 c.c. of 0.02 N hydrochloric acid are added (this is about 2 c.c. of 0.02 N acid in excess of the bicarbonate normally present). In order to remove the carbon dioxide set free by the acid, the flask is shaken vigorously with a rotary motion, so that the solution is whirled in a thin layer about the inner wall. One minute of this treatment is sufficient to remove carbonic acid so completely that not enough is left to affect the results measurably. The solution is now poured as completely as possible into a 50 c.c. Erlenmeyer flask and the walls of the larger flask are rinsed with 20 c.c. of water. The water is measured within 1 c.c. in a cylinder, and approximately a third is used for each of three washings.

When the solution, measuring about 26 c.c., has been transferred to the 50 c.c. flask, 0.3 c.c. of a 0.1 per cent solution of

[1] Van Slyke, Stillman and Cullen: Jour. Biol. Chem., 1919, 38, 167.

neutral red (dissolved in 50 per cent alcohol) is added. 0.02 N *carbonate-free* sodium hydroxide is then run in from a burette (preferably but not necessarily a micro-burette) until the color of the solution matches that of 29 c.c. of a standard phosphate solution, of pH 7.4, contained in a similar 50 c.c. flask.

In place of neutral red 0.3 c.c. of a 0.04 per cent solution of phenolsulfonephthalein may be used as indicator, and gives an end-point slightly more easy to distinguish than that of neutral red. When phenolsulfonephthalein is used, however, the standard solution must be of pH 7.2 instead of 7.4. The reason for this is that when phenolsulfonephthalein is added to a solution containing serum proteins, the pH is considerably higher than that estimated by colorimetric comparison with standard phosphate solutions. The indicator suffers from a protein error. Under the conditions of the analysis here described, however, the error is quite constant at 0.2 to 0.3 pH, i.e., if the color matches that of a standard solution of pH 7.4, the actual pH in the solution under examination is about 7.7. If a standard solution of pH 7.2 is used, however, the titration runs to the same point obtained with neutral red.

For the titrations there is used a 3 c.c. micro-burette of the model devised for use with the blood sugar method of Bang. Such a burette is desirable but not absolutely necessary. An ordinary 50 c.c. burette divided into 0.1 c.c. divisions may be employed.

The End-point.—With both indicators, a peculiar phenomenon occurs as the end-point is approached. Each drop appears to change the color past the end-point, but within a few seconds the color shifts back, and it is seen that at least another drop is needed before the genuine end-point is reached. Consequently, the final color comparison should not be made until at least thirty seconds after the last drop of 0.02 N sodium hydroxide has been added. Because of this behavior, as well as the character of the color change, it is well, particularly with neutral red, to overrun the end-point by a drop, rather than stop short of it when in doubt.

Calculation of Results.—The number of c.c. of 0.02 N sodium hydroxide used in the titration is subtracted from the number required to neutralize to the same indicator 5 c.c. of the 0.02 N hydrochloric acid used. This number is, of course, approximately

5, but it usually varies slightly from that because of difference in the factors of acid and alkali and because of the calibration error of the 5 c.c. pipette used for measuring the acid. Consequently the maximum accuracy is obtained by performing a preliminary titration on 5 c.c. of the acid plus 20 c.c. of the distilled water, using the same pipette, indicator, and end-point as in the plasma titration.

The following is a typical calculation on a normal human plasma:

0.02 N NaOH = HCl added.....	5.09 c.c.
0.02 N NaOH taken in titration.....	2.03 c.c.
0.02 M $NaHCO_3$ in 2 c.c. plasma or 0.01 M $NaHCO_3$ in 1 c.c. plasma.....	3.06 c.c.

$3.06 \div 100 = 0.0306$ = molecular concentration of $NaHCO_3$ in plasma.

$3.06 \times 22.4 = 68.5$ volume per cent CO_2 bound as bicarbonate in the plasma.

Since the titration result represents c.c. of 0.01 M $NaHCO_3$ per c.c. of plasma, it is transformed into terms of molecular concentration of $NaHCO_3$ in the plasma merely by dividing by 100.

For the sake of comparison with results of bicarbonate determination by the CO_2 method (Van Slyke and Cullen), the molecular concentration is multiplied by 2240 in order to give results in terms of c.c. of CO_2 per 100 c.c. of plasma. According to the gas laws the amount of CO_2 contained in a M carbonate solution is 22,400 c.c. per liter (measured as CO_2 gas at 0°, 760 mm.) or 2240 c.c. of gas per 100 c.c. of solution. Hence multiplying the bicarbonate molecular concentration by 2240, or multiplying the c.c. of 0.02 N acid used in the titration by 22.4, gives the volume per cent of bicarbonate CO_2 in the plasma.

Inversely, of course, dividing the volume per cent of CO_2, as determined by Van Slyke and Cullen, by 2240 transforms the CO_2 figures into terms of molecular concentration.

Solutions. *The Standard 0.02 N Sodium Hydroxide.*—The 0.02 N NaOH as a basis for the determination must, in order to maintain its value, be protected from contact with atmospheric carbon dioxide and from glass. Even standing over night in a burette of soft glass is likely to result in the solution of enough alkali to raise the titration value of the standard solution. The standard alkali should be kept in paraffined bottles, and the burette filled with fresh solution each day that it is used.

In order to obtain a carbonate-free alkali solution, use is made of the well-known expedient of first dissolving the NaOH in an equal weight of water. Sodium carbonate is insoluble in such a concentrated alkali solution and settles to the bottom. 5.5 c.c. of the clear supernatant solution diluted to 5 liters yields an approximately 0.02 N solution which is standardized by titration with neutral red against 0.02 N HCl. In performing the titration it is preferable to run the acid into the alkali, thus titrating from the yellow alkaline color to the acid red. The color change in this direction occurs without the time lag observed when alkali is added to acid.

The Standard Solutions of pH 7.2 and 7.4.—The standard solutions of pH 7.2 and 7.4 may be made as follows: M/20 solutions being obtained. The M/5 KH_2PO_4 contains 27.23 gm. of KH_2PO_4 per liter.

pH	M/5 KH_2PO_4 c.c.	N/5 NaOH c.c.	
7.2	50	35.0	Dilute to 200 c.c.
7.4	50	39.5	Dilute to 200 c.c.

They may also be made by Sörensen's method from KH_2PO_4 and Na_2HPO_4 as follows: the phosphate concentration being M/15.

pH	Na_2HPO_4 gm.	KH_2PO_4 gm.	
7.2	6.89	2.47	Dilute to 1 liter.
7.4	7.72	1.67	Dilute to 1 liter.

If crystalline $Na_2HPO_4 \cdot 2H_2O$ is used instead of the anhydrous Na_2HPO_4, the amounts are increased to 8.66 and 9.67 gms. for the solutions of pH 7.2 and 7.4 respectively.

The plasma solution becomes somewhat turbid, but the turbidity partially clears up as the end-point is approached, and the latter can be determined within one drop of the 0.02 N NaOH.

MICRO-TITRATION [1]

When it is desirable to employ only small amounts of plasma, 0.400 c.c. may be transferred to a test-tube of about 20 mm. diameter, and 1 c.c. of 0.002 N HCl added. The tube is laid in a nearly horizontal position, so that the layer of liquid extends for about 10 cm. along the lower side. The tube is then rotated or rolled back and forth vigorously, but not so roughly as to cause foaming, for not less than 1.5 minutes, in order to cause the carbon dioxide to escape. Three drops of indicator are then added, and the solution is titrated in the test-tube with 0.004 N NaOH to a pH of 7.4, using as a color standard a like volume of phosphate solution in a similar tube.

The 0.004 N NaOH is so rapidly altered by contact with either glass or atmospheric carbon dioxide that it is advisable to make it fresh for each series of analyses by diluting 10 c.c. of 0.1 N NaOH to 250 c.c. with distilled water that has been freed of CO_2 either by boiling or by shaking in an evacuated flask. The control titration of the 0.004 N NaOH against the 1 c.c. of 0.02 N HCl should be performed immediately before the plasma titration.

The calculation is the same as in the larger titration. The number of c.c. of 0.004 N NaOH used is subtracted from the number of c.c. (approximately 5) required to neutralize the HCl in the control titration. The difference divided by 100 represents the molecular concentration of bicarbonate in the plasma, while the difference multiplied by 22.4 indicates the volume per cent of bicarbonate CO_2.

With care in the calibration of pipettes, and especially in the control of the 0.004 N NaOH, results nearly and perhaps quite as accurate as in the larger titration appear attainable.

ALKALI RESERVE

Indirect Method. Alveolar Carbon Dioxide Tension Marriott's Method [2]

While this method is open to criticism because of the liability of error in the collection of the sample and, more fundamentally,

[1] Jour. Biol. Chem., 1919, 38, 177.

[2] Marriott: Jour. Am. Med. Assn., 1916, 66, 1594.

because of various factors (psychical, etc.) other than acidosis which may influence the carbon dioxide tension, nevertheless, it is of considerable value and has been rather widely adopted for clinical use.

Principle.—By rebreathing air under certain definite conditions a sample is obtained whose carbon dioxide tension is virtually that of venous blood. The method of analysis of this sample depends on the fact that if a current of air containing carbon dioxide is passed through a solution of sodium carbonate or bicarbonate until the solution is saturated, the final solution will contain sodium bicarbonate and dissolved carbon dioxide. The reaction of such a solution will depend on the relative amounts of the alkaline bicarbonate and the acid carbon dioxide present. This, in turn, will depend on the tension of carbon dioxide in the air with which the mixture has been saturated and will be independent of the volume of air blown through, provided saturation has once been attained. High tensions of carbon dioxide change the reaction of the solution toward the acid side. Low tensions have the reverse effect; hence the reaction of such a solution is a measure of the tension of carbon dioxide in the air with which it has been saturated. A suitable indicator is added to the solution and its reaction (after the passage of the alveolar air) is determined by comparison with a set of suitable standards.

Procedure.—Collection of the alveolar air. The method of collection is essentially that of Plesch, as modified by Higgins. A rubber bag of approximately 1500 c.c. capacity (A basket-ball bladder or a hot-water bag answers very well. If the latter is used, the neck may be closed by a rubber stopper carrying a short glass tube 3/8 inch in internal diameter) is connected by means of a short rubber tube to a glass mouth-piece. (An ordinary piece of glass tubing with rounded edges, $1\frac{1}{2}$ inches long and 3/8 inch in internal diameter.) About 600 c.c. of air are blown into the bag with an atomizer bulb, and the rubber tube clamped off by a pinchcock. The subject should be at rest and breathing naturally (especially to be guarded against is a deep, voluntary inspiration just before the collection begins, as this causes too low a determination). At the end of a normal expiration, the subject takes the tube in his mouth; the pinchcock is released and the subject's nose closed by the observer. The subject breathes back and forth from the bag four times in twenty seconds, emptying

the bag at each inspiration. The observer should indicate when to breathe in and out. Breathing more frequently will not greatly alter the results. At the end of twenty seconds, the tube is clamped off and the air analyzed. The analysis should be carried out within three minutes' time, as carbon dioxide rapidly escapes through rubber.

Analysis of Sample.—In analyzing a sample of air, about 2 or 3 c.c. of the standard bicarbonate solution are poured into a clean test-tube of the same diameter as the tubes containing standard phosphate solutions, but from 100 to 150 mm. long. Air from the bag is then blown through the solution by means of a glass tube drawn out to a fine capillary point, until the solution is saturated, as shown by the fact that no further color change occurs. (If the operator first blows his own breath through the solution so as to bring it into approximate equilibrium with alveolar air, saturation may be accomplished with as little as 100 c.c. of air from the bag, blown through during thirty seconds. The same bicarbonate solutions may be used for repeated determinations.) The tube is stoppered and the color immediately compared with that in the standard tubes. By interpolation, one can readily read to millimeters. Color changes are not quite so sharp above 35 mm. as at the lower end of the scale, but here changes are of less significance. In making the color comparisons the solution being compared is placed between the two standards which it most nearly matches. When there is doubt as to whether the color of the solution is higher or lower than one of the standards, changing the order in which the tubes are placed in the comparison box will generally make the relationship clear.

The standard solutions described are so prepared as to give correct results when the determination is carried out at a temperature of from 20° to 25° C. (from 68° to 77° F.). When the room temperature is considerably higher or lower than these points it is advisable to immerse the tubes in water at approximately 25° C. during the blowing. They may be removed from the water for the color comparison, however, provided this is quickly made. The differences due to ranges of temperature occurring under ordinary circumstances are practically negligible. (No correction for barometric pressure is required as from the nature of the determination, barometric fluctuations are self-corrective. Variations in the temperature of the subject are never

great enough to affect the value as much as 1 mm. and therefore may be neglected.)

Calculation.—The standard tubes are marked to indicate the carbon dioxide tension in millimeters of mercury, and the readings can be estimated to about 2 mm.

ALKALI RESERVE

Indirect Method. Alveolar Carbon Dioxide Tension
Fridericia's Method [1]

Principle.—The method of determination is based upon the absorption, by means of potassium hydroxide, of the carbon dioxide from a known amount of alveolar air. The apparatus is so graduated that the decrease is volume may be read in per cent.

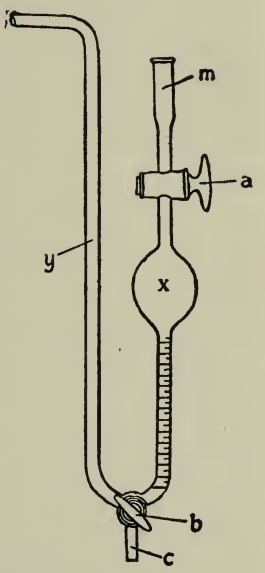

Fig. 12.—Fridericia Apparatus. (Hawk.)

Procedure.—The subject must sit quietly in a chair and breathe naturally, holding the apparatus (shown in Fig. 12) in front of him with the cock *a* open and *b* in a position connecting *x* with *y*. (It is especially important to caution the subject against the very natural inclination to take an abnormally deep inspiration just before blowing through the apparatus, and also to see that, in seeking to avoid this fault, the breath is not held just before the sample is taken.) After taking a normal inspiration he places the mouthpiece *m* into his mouth and blows as hard and as quickly as possible through the apparatus, thus washing it out and leaving it filled with alveolar air. The cock *a* is at once closed and the whole apparatus is immersed in water for five minutes. By this means the alveolar air in *x* and *y* is cooled to a temperature which remains constant throughout the experiment, and the contraction in volume causes the alveolar air in

[1] Fridericia: Hospitalstidende, Copenhagen, 1914, 57, 585. Poulton: Brit. Med. Jour., 1915, 2, 392.

the lower part of y to be drawn back into x. (Any diffusion with the outside air at the top of y will not reach to the bottom of the tube owing to its length.) At the end of five minutes the cock b is turned so as to connect y with c, thus closing x, which then contains exactly 100 c.c. of alveolar air at atmospheric pressure and at the temperature of the water in which the apparatus is immersed, this temperature remaining constant throughout the determination. The apparatus is removed from the water, the tube c is placed beneath the surface of some 10 per cent sodium hydroxide solution, some of the alkali is drawn up into y, the apparatus is held in such a position that y is rather depressed in order to prevent the escape of small bubbles of gas from x, the cock is turned so as to connect y with x, and some of the alkali is forced into x. The cock b is at once turned, closing x and connecting y with c through which the remainder of the alkali is allowed to flow. The apparatus is inverted several times during the course of half a minute which is sufficient time for the absorption of all the carbon dioxide. It is then returned to the water which rises through c into y, after which b is turned to connect y and x and the whole is allowed to remain for five minutes to again equalize the temperature. It is then raised rapidly until the water in the graduated portion of x is at the same level as the water outside the apparatus, i.e., when the gas within the tube x is under atmospheric pressure.

Calculation.—The reading of the bottom of the meniscus of the fluid in x is taken and represents, without any further calculation or correction, the percentage of carbon dioxide in the alveolar air. If it is desired to express this percentage as the partial pressure of CO_2 in millimeters of mercury it is multiplied by a figure 40 mm. less than the prevailing barometric pressure; e.g., if the reading of the apparatus is 5.5 then the calculation will be as follows: 5.5 per cent CO_2 or $0.055 \times (760-40)$. This 40 mm. is the tension of water vapor in the lungs at body temperature. It is sufficient for clinical purposes to use the mean barometric pressure of the locality, neglecting the daily variations.

ALKALI RESERVE

Indirect Method. Index of Acid Excretion in Urine
Method of Fitz and Van Slyke [1]

Principle.—The method depends upon the determination of the rate of excretion of acid (NH_3+titratable acid) from which the plasma carbon dioxide capacity is calculated.

Procedure.—Collect the urine for twenty-four-hours (or if desired for a period of 1 or 2 hours during which the subject ingests neither food nor water). In the latter case the urine collection should not be too soon after a meal. Measure, carefully, the volume of the urine and determine its ammonia content according to the method given on page 42 and the titratable acid according to the method given on page 21. Obtain the body weight of the patient.

Calculation.—The plasma bicarbonate may be calculated by substitution in the following equation:

$$\text{Plasma Carbon Dioxide Capacity} = 80 - 5\sqrt{\frac{D}{W}}.$$

D = Rate of excretion per twenty-four hours.
W = body weight in kilograms.

(The value 80 represents the maximum normal value of plasma bicarbonate. Under such a condition the titratable acid and ammonia excretion tend to approach zero.)

The value D is equal to the product VC, in which V is the twenty-four-hour volume (if the urine is collected for only one or two hours its volume is, of course, multiplied by 24 or 12 as the case may be) expressed in liters, and C the sum of the ammonia (expressed as c.c. of N/10 NH_3 per liter of urine) plus the titratable acid (expressed as c.c. of N/10 acid per liter of urine). For practical purposes the acid excretion may, without going through the calculation of the formula, be interpreted directly into terms of clinical severity of acidosis, as indicated in the table on page 144, e.g., an excretion exceeding 27 c.c. of N/10 ammonia plus titratable acid per kilo indicates acidosis, which usually becomes critical in severity if the excretion approaches 100 c.c. per kilo.

[1] Fitz and Van Slyke: Jour. Biol. Chem., 1917, 30, 389. Van Slyke: Ibid. 1918, 33, 271. Barnett: Ibid., 1918, 33, 267.

ALKALI TOLERANCE[1]

This method is quite reliable for proving the absence of acidosis, but is not particularly dependable for showing either the presence or the degree of acidosis when it exists. This seems to be due in part to the fact that in conditions associated with acidosis the power of the kidney for excretion of alkalies may be markedly impaired.

THE RELATIONSHIP OF THE PLASMA BICARBONATE TO ACID DIOXIDE

Corresponding results of

Condition of subject	Actual bicarbonate reserve Plasma bicarbonate CO_2 reduced to 0°, 760 mm. Vol. per cent	24-Hour Excretion* of N/10 Acid+NH_3	
		(*a*) c.c. per kg. (*b*) Approximate c.c. per 60 kg. person, c.c.	Reliability in diabetes
Normal resting adult. Extreme limits of bicarbonate reserve..	80–53	(*a*) 0–27 (*b*) 0–1600	Good
Mild acidosis, no pronounced symptoms.	53–40	(*a*) 27–65 (*b*) 1600–4000	Good†
Moderate to severe acidosis. Symptoms may be apparent.	40–30	(*a*) 65–100 (*b*) 4000–6000	Liable to considerable error in either direction.†
Severe acidosis..... Symptoms of acid intoxication.....	Below 30	(*a*) Over 100 (*b*) Over 6000	

* Measured either in twenty-four-hour urine or on specimen from shorter period calculated to twenty-four-hour basis.

† After bicarbonate administration likely to indicate more acidosis than is present.

[1] Sellards: Bull. Johns Hopkins Hosp., 1912, 23, 289; Palmer and Henderson: Arch. Int. Med., 1913, 12, 153; Palmer and Van Slyke: Jour. Biol. Chem., 1917, 32, 499.

Principle.—Sodium bicarbonate is administered in small amounts, either by mouth or intravenously until the reaction of the urine changes from acid to alkalies. The amount of bicarbonate is then noted.

Procedure.—Give (by mouth) 5 gms. of sodium bicarbonate in 100 c.c. of water to the subject under examination. Repeat every half hour until the total bicarbonate administration is equivalent to 0.5 gm. per kilogram of body weight unless the urine becomes alkaline before that time. In case the urine does not

EXCRETION, ALKALI TOLERANCE, AND ALVEOLAR CARBON TENSION

indirect tests for acidosis

Carbon Dioxide of Alveolar Air.		Sodium Bicarbonate Required to Turn Urine Alkaline	
(*a*) Mm. tension. (*b*) Approximate per cent	Reliability in diabetes	(*a*) Gm. per kg.‡ (*b*) Approximate gm. for a 60 kg. person	Reliability in diabetes
(*a*) 53–35 mm. (*b*) 6.8–4.7 per cent	May indicate some acidosis in its absence.	(*a*) 0–0.5 (*b*) 0–30	May indicate acidosis in its absence
(*a*) 35–27 mm. (*b*) 4.7–3.6 per cent	May indicate more acidosis than is present.	(*a*) 0.5–0.8 (*b*) 30–50	May indicate much more acidosis than is present.
(*a*) 27–20 mm. (*b*) 3.6–2.7 per cent	Good	(*a*) 0.8–1.1 (*b*) 50–65	
(*a*) Below 20 mm. (*b*) 2.7 per cent	Good	(*a*) Over 1.1 (*b*) Over 65	

‡ The figures tabulated in this column also indicate the doses of bicarbonate necessary to restore the alkali reserve to normal from acidosis of the severity indicated by the corresponding plasma CO_2 figures in the first column.

become alkaline with the above bicarbonate ingestion, continue the administration of the alkali until the urine shows an alkaline reaction. (Because of the likelihood of producing a condition of

alkalosis it is advisable not to continue the administration of bicarbonate without evidence from blood analysis showing an alkali deficit.) The urine should be voided by the subject before each administration of bicarbonate. Test each specimen of urine with litmus, boiling those samples which are only faintly acid so that any bicarbonate present will be converted to carbonate. Note the number of grams of bicarbonate necessary to produce an alkaline urine.

NEPHELOMETRIC METHODS [1]

The Nephelometer.—The nephelometer is an instrument for measuring the density of precipitates and thus determining the amount of any substance which can be obtained in the form of a suitable suspension. It is somewhat similar in form and principle to a colorimeter. It differs from the latter in that the light which reaches the eye is not transmitted light, which, on the contrary, is excluded, but light reflected from the particles of the suspension. The brightness of the two fields is compared instead of their colors. It is adapted particularly for the determination of substances that in very dilute solution may be precipitated in the form of suspensions which do not agglutinate appreciably in the time required for making readings (ten to twenty minutes). The method has been adapted to the determination of proteins in digestion mixtures, milk, urine, etc.; nucleic acids; chlorides, phosphates, and phosphatides in blood, etc.; fats in milk, blood, etc.; acetone bodies in urine and blood; uric acid and purine bases; ammonia; calcium; silver, etc., and is continually finding new applications. It is possible to determine very minute amounts of substances, entirely outside of the range of gravimetric methods of analysis, and hence the procedure may be used where the amount of material is very limited. If properly carried out the limits of error of the method are not greater than those of the colorimetric methods commonly used.

Nephelometric Calculations.—The amounts of precipitate in solutions examined nephelometrically is not exactly inversely proportional to the readings of the scale. When the concentration of the unknown and of the standard are within 10 per cent of each

[1] Hawk: Practical Physiological Chemistry, Sixth Edition, 1918.

other (or within about 20 per cent if the readings are made at depths as great as 50 to 60 mm.) accurate results may, however, be obtained directly. If the variations are greater than this a correction is necessary. Kober has proposed an equation to supply this correction and thus make possible very accurate work under conditions of moderate variations of concentration. The equation is as follows:

$$y=\frac{s}{x}-\frac{(1-x)sk}{x^2}$$

or

$$x=\frac{s+sk+\sqrt{(s+sk)^2-4sky}}{2y}$$

where y = height of unknown solution, on the left side of the instrument, when standard solution is kept on the right side at a definite height, s = height of standard solution on the left side and x = the ratio of the concentrations of the two solutions.

$k=\frac{K}{s}$, where K = a constant, obtained by substitution of standardization values of s, y, and x. The instrument should be checked up for each series of analyses by reading the standard against itself and determining the potential height of the standard solution by reading the scale on the left side when the solution on the right side is kept at a definite height, and the two are matched.

DUBOSCQ COLORIMETER TRANSFORMED INTO A NEPHELOMETER

(*Bloor*) [1]

The following description for changing the Duboscq colorimeter into a nephelometer applies only to the later type of colorimeter, that is, the cups move and the prisms are stationary. The extra parts necessary are supplied in an improved form by the International Equipment Company of Cambridge, Mass. By the use of these parts the change may be quickly made as follows:[2] Unscrew the movable glass prisms of the colorimeter, slip the

[1] Bloor: Jour. Biol. Chem., 1915, 22, 145.

[2] Folin: A Laboratory Manual of Biological Chemistry, 1919.

brass collars for the nephelometer tubes into place, and fasten on the plate from which the prisms were removed. Slip the movable jackets into the holes in the cup supports, and after pushing the nephelometer tubes into place in the collars, the instrument is ready for use. A darkened room and a light-tight box for the light are necessary. The box should be about 48 cm. long, 32 cm. high, and 20 cm. wide for the ordinary colorimeter. It should contain a bracket at one end to support the light (a 50-watt Mazda) at the height of the nephelometer tubes, and a stop at the other end, against which the instrument may be pushed and so placed that the nephelometer tubes are about 30 cm. from the light. A slot in the top of the box to receive the telescope of the instrument and a dark curtain to cover the end of the box after the instrument is pushed into place complete the equipment of the box. All exposed parts should be painted a dull black.

Since the readings obtained from suspensions of different strength are not exactly proportional to the amount of precipitate present, it is necessary to calibrate the instrument for different strengths and make corrections accordingly. If, however, the solution to be tested is within 25 per cent of the value of the standard, no correction is necessary.

ACETONE BODIES

Nephelometric Methods of Marriott [1]

Principle.—Acetone in very small amounts forms a cloudy solution with the Scott-Wilson reagent which may be read nephelometrically. By this method it is possible to make a complete analysis for acetone and diacetic acid and hydroxybutyric acid in from 2 to 5 c.c. of blood.

Procedure.—Two to 5 c.c. of blood drawn from a superficial arm vein by means of a sterile syringe are run into a small weighed flask containing 50 c.c. of 0.5 per cent potassium oxalate solution. The flask is reweighed. The diluted blood is run into 100 c.c. of boiling water acidified with 1 c.c. of glacial acetic acid contained in an 800 c.c. Kjeldahl distilling flask and the procedure is then carried out as described in the Marriott-Scott-Wilson methods for (*a*) acetone and diacetic acid and (*b*) β-hydroxybutyric acid

[1] Marriott: Jour. Biol. Chem., 1913, 16, 289.

(see below). (Commercial varieties of acetic acid frequently contain substances which behave like acetone. Blank determinations should always be made and corrections made accordingly.) The precipitate in the mercury reagent is, however, estimated nephelometrically. In this case the distillate which should measure 75-100 c.c. is allowed to stand half an hour, then transferred to a graduated cylinder and diluted until an opalescence that can be conveniently read is obtained. The turbidity occasioned by 0.05 mg. of acetone diluted to 100 c.c. is a convenient strength for this purpose, although considerably larger or smaller amounts give good results. With heavy opalescence it is desirable after diluting to a certain volume, say 250 c.c. to remove an aliquot portion with a pipette and dilute this appropriately. A solution containing a known amount of acetone (a convenient stock solution contains about 0.03 mg. acetone per c.c.) is distilled into an excess of reagent (the solution cannot be added directly to the reagent as a lower result is obtained than when distilled) and this distillate which is to be used as the standard is diluted as above. Read in the nephelometer against this standard, taking the readings as quickly as possible after filling the tubes as the suspension settles slowly.

If the unknown suspension is diluted so as to be not more than 20 per cent different from the standard and if comparisons are made in considerable depths of solution (50 to 60 mm.) no corrections are necessary. If the two agree within 10 per cent accurate comparison may be made at less depths. If divergences are greater and accurate results are desired, Kober's correction equation must be used (see discussion of nephelometer, page 146).

ACETONE, ACETOACETIC ACID AND β-HYDROXYBUTYRIC ACID

Marriott-Scott-Wilson Method [1]

(a) **Acetone and Acetoacetic Acid.**—Draw 10 c.c. of blood from a superficial vein by a sterile graduated syringe and run it into about 40 c.c. of 0.5 per cent potassium oxalate solution. Fit up a Kjeldahl distillation apparatus, using an 800 c.c. flask,

[1] Scott-Wilson: Jour. of Physiol., 1911, 42, 444. Marriott: Jour. Biol. Chem., 1913, 16, 295.

provided with a dropping funnel, the delivery tube of the condenser dipping beneath the surface of the water in a receiving flask. Introduce into the Kjeldahl flask 100 c.c. of water and 1 c.c. of glacial acetic acid. Bring the acidified water to a boil and then run the diluted blood in slowly through the dropping funnel.

Boil for thirty minutes after the last blood is run in. (If β-oxybutyric acid is to be determined the residue in the Kjeldahl flask should be kept and treated as outlined in the latter part of this procedure.) To the distillate add a little dilute sulphuric acid and redistill. To this distillate add 20 c.c. of hydrogen peroxide solution and a slight excess of alkali and redistill again. The final distillate is caught in small Erlenmeyer flasks containing an excess of the Scott-Wilson "acetone reagent" which has been recently filtered. The delivery tube must dip under the surface of the liquid. It is not necessary to distill more than ten minutes to get off all the acetone. Allow to stand for ten to fifteen minutes. Filter through an asbestos mat (filter paper cannot be used as the strong alkali quickly attacks it) in a separable bottom Gooch crucible. Clear filtrates are more readily obtained if the pores of the filter have been partly closed by filtering through it a suspension of talcum powder in water. If the first portions of the filtrate are turbid, refilter. Wash the precipitate with cold water until the washings are free from silver.

Preparation of the Scott-Wilson Acetone Reagent.—The reagent is made up as follows: Mercuric cyanide, 10 gms.; sodium hydroxide, 180 gms.; water, 1200 c.c. The solution is agitated in a flask and 400 c.c. of a 0.7268 per cent solution of silver nitrate slowly run in. At least 30 c.c. of the reagent must be taken for each milligram of acetone present.

(b) **Determination of β-Hydroxybutyric Acid.**—The residue in the Kjeldahl flask from the above determination is used in the determination of β-hydroxybutyric acid. While still hot, precipitate it with about 8 c.c. of 10 per cent sodium carbonate, boil a moment, filter on a Büchner funnel and wash with hot water. To the clear filtrate add 15 c.c. of basic lead acetate (U.S.P.) and 10 c.c. of strong ammonia and make to definite volume (150 c.c.) with water. Allow the precipitate to settle and then filter off on a dry, folded filter. Take an aliquot of the clear filtrate (about 125 c.c.) and boil it to expel the greater part of the ammonia. Cool and add dilute sulphuric acid to precipitate the

excess of lead as sulphate and filter. Add 10 c.c. of 50 per cent sulphuric acid and transfer the whole to a Kjeldahl flask provided with a dropping funnel. The contents of the flask are distilled and a solution of potassium bichromate is run in from the dropping funnel at such a rate that the liquid always retains some yellow color and the volume remains at about 100 c.c. It is rarely necessary to add more than about 0.1 gram of bichromate and an excess is to be avoided. Slow distillation is continued for two hours and about 100 c.c. of distillate is collected. The tip of the delivery tube must always remain under the surface of the water in the receiving flask. Add 20 c.c. of hydrogen peroxide, make slightly alkaline with NaOH and distill again. Catch the distillate in small Erlenmeyer flasks containing an excess of the Scott-Wilson acetone reagent (at least 30 c.c. for each milligram of acetone) and determine the acetone according to the procedure outlined in the preceding method for acetone and diacetic-acid.

FAT

Nephelometric Method of Bloor [1]

Principle.—The protein is precipitated with alcohol and ether and the fatty acid in the extract determined nephelometrically after saponification.

Procedure.—*Extraction.*—About 2 c.c. of blood are drawn from the vein with a graduated syringe and run at once with stirring into a weighed graduated flask containing about 40 volumes of a mixture of 3 parts alcohol and 1 part ether. After again weighing to find the weight of blood added, the solution is raised to boiling in a water-bath, cooled under the tap, made to volume with alcohol-ether mixture, mixed and filtered. The filtrate is water clear and almost colorless.

Determination.—From 5 to 20 c.c. of the extract (containing about 2 mg. of fat) are measured with a pipette into a small beaker and saponified by evaporating nearly but not quite to dryness with 2 c.c. of N/1 sodium ethylate. The residue is heated just to boiling after the addition of 5 c.c. of alcohol-ether, and 50 c.c. of distilled water are added.

[1] Bloor: Jour. Biol. Chem., 1914, 17, 377; 1915, 23, 317.

A similar solution of the standard is prepared by adding 5 c.c. of the standard fatty acid solution from a pipette with stirring to 50 c.c. of distilled water. To the standard and to the test solutions are added simultaneously from pipettes and with stirring 10 c.c. portions of dilute (1:3) hydrochloric acid and the solutions allowed to stand for five minutes, after which they are transferred to the comparison tubes of the nephelometer. Several readings should be taken and averaged. The standard tube should always be on the same side. See discussion of nephelometer (page 146) for details as to reading. The results represent the amount of total fat (fatty acids and cholesterol) in the blood, expressed as oleic acid. The fat of the corpuscles is not completely extracted, and it should be borne in mind that other lipoids as cholesterol are included in the results. Cholesterol may be determined separately and subtracted from the result for total fat. It may also be determined in a part of the blood extract as prepared above by a modified Autenrieth-Funk procedure (see page 156). Methods have also been devised for the determination of the phosphatides of blood (see page 171).

BLOOD ACETONE BODIES

Determination of Acetone Bodies. Method of Van Slyke and Fitz [1]

Principle.—The principle is identical with that for the determination of acetone bodies in urine after the removal of the proteins from the blood.

Procedure.—*Whole Blood.*—Ten c.c. of whole blood are diluted with about 100 c.c. of water in a 250 c.c. flask, and 20 c.c. of the 10 per cent mercuric sulphate are added. (73 gms. of red mercuric oxide dissolved in 1 liter of 4 N sulphuric acid.) The solution is shaken for a moment, until the protein coagulates, and is then diluted with water up to the 250 c.c. mark. After fifteen minutes or longer it is filtered through a dry folded filter. If the first drops are cloudy they are passed through a second time. The filtrate has a slight pink tinge, but the substance responsible for it does not precipitate when boiled with mercuric sulphate, nor otherwise interfere with any of the acetone body determinations.

[1] Van Slyke and Fitz: Jour. Biol. Chem., 1917, 32, 495.

If the blood is diluted with much more than 10 volumes of water before the mercury is added, coagulation of the proteins is considerably slower, hence the reason for not completing the dilution until after the coagulation has occurred.

Plasma or Serum.—Eight c.c. of oxalate plasma or of serum are diluted in a 200 c.c. flask with 50 c.c. of water and 15 c.c. of the mercuric sulphate are added. The flask is shaken for a moment, until the fine precipitate which first forms has flocculated, and is then filled to the mark with water. After standing fifteen minutes or longer the solution is filtered.

Determinations. — For determination of acetone plus acetoacetic acid, of β-hydroxybutyric acid, or of the total acetone bodies together, 125 c.c. of the filtrate, equivalent to 5 c.c. of either blood or plasma, may be treated exactly as the 25 c.c. of urine filtrate plus 100 c.c. of water in urine analysis. (See urine method.)

It should be noted, however, that the precipitate should be filtered soon after the period of boiling is ended. If the mixture is allowed to cool and stand for several hours, several mg. of flocculent precipitate of indefinite origin may form and cause a plus error of appreciable magnitude in the results.

FACTORS FOR CALCULATING RESULTS WHEN FILTRATE EQUIVALENT TO 5 c.c. OF BLOOD IS USED FOR DETERMINATION

Determination Performed	Acetone Bodies, Calculated as Gm. of Acetone per Liter of Blood, Indicated By	
	1 gm. of precipitate	1 c.c. of 0.2 M KI solution
Total acetone bodies	12.8	0.161
β-hydroxybutyric acid	13.2 (14.0) *	0.172 (0.183)*
Acetone plus acetoacetic acid	10.0	0.130

* (These factors are used when β-hydroxybutyric acid is determined in the filtrate from the precipitated acetone and acetoacetic acid as described above. In this case the amount of filtrate taken for the β-acid determination is equivalent to only $\frac{160}{170}$ of 5 c.c. of blood, and the factor must be correspondingly increased.)

In case it is desired to determine separately the acetone plus acetoacetic acid and the hydroxybutyric acid in a single sample of blood, this may be done by first precipitating the preformed acetone plus that from acetoacetic acid, and then determining the hydroxybutyric acid in the filtrate. The preformed acetone plus that from acetoacetic acid is precipitated exactly as in urine analysis. The filtrate from the mercury-acetone precipitate is received into a dry flask. After as much of the solution as possible has been filtered through, and before any wash water has been used, 160 c.c. of the filtrate, equivalent to $\frac{160}{170} \times 5$ c.c. of blood, are placed in a 500 c.c. Erlenmeyer flask, heated to boiling under a reflux condenser, and 5 c.c. of 5 per cent potassium dichromate solution are added through the condenser. The rest of the hydroxybutyric acid determination is carried out as described for urine from the point where the dichromate is added.

To calculate the acetone bodies as β-hydroxybutyric acid instead of as acetone, multiply the above factors by 1.793; to calculate molecular concentration, divide the factors by 58.

Normal blood when analyzed as described for total acetone bodies yield only 1 or 2 mg. of precipitate, equivalent to 0.013 to 0.026 gm. of acetone per liter. In diabetics as much as 2.5 gms. of acetone bodies calculated as acetone has been observed, while patients under ordinarily good control show 0.1 to 0.4 gm.

CHOLESTEROL IN BLOOD OR BLOOD SERUM

Method of Bloor[1]

Principle.—The protein is precipitated with a mixture of alcohol and ether and the cholesterol in the filtrate is estimated colorimetrically against a standard solution of cholesterol.

Procedure.—*Preparation of the Sample.*—3 c.c. of whole blood, plasma, or serum are run slowly (a slow stream of drops) from a pipette into about 75 c.c. of a mixture of redistilled alcohol and ether (3 parts alcohol, 1 part ether) in a 100 c.c. graduated flask. The contents of the flask should be kept in motion during the process so that there is no clumping of the precipitated material. The contents of the flask are raised to boiling by immersion in a water bath (with constant shaking to avoid superheating), cooled

[1] Bloor: Jour. Biol. Chem., 1916, 24, 227.

to room temperature, filled to the mark with alcohol-ether, mixed, and filtered. The filtered liquid if placed in a tightly stoppered bottle in the dark will keep unchanged for a considerable time so that if it is not convenient to complete the determination at once, the sample may be carried to the above stage and left till a more suitable time.

By running the blood slowly into the large quantity of alcohol-ether, as above, the protein material is precipitated in finely divided form and under these conditions the short heating combined with the great excess of solvent is adequate for complete extraction of serum or plasma. The extraction, while not quite so complete in the case of whole blood, is believed to be better (because of the higher values obtained) than that obtained by any other method in use at the present time.

The determination of fat (see page 151) may be made upon the same sample of blood plasma or serum.

Determination.—10 c.c. of the alcohol-ether extract are measured into a small flat-bottomed beaker and evaporated *just* to dryness on a water bath or electric stove. Any heating after dryness is reached produces a brownish color which passes into the choloroform and renders the subsequent determination difficult or impossible. The cholesterol is extracted [1] from the dry residue by boiling out three or four times with successive small portions of chloroform and decanting into a 10 c.c. glass-stoppered, graduated cylinder after cooling (5 c.c. or less) are then made up to 5 c.c. The solution should be colorless but not necessarily clear, since the slight turbidity clears up on adding the reagents.

5 c.c. of a standard cholesterol solution in chloroform [2] (containing 0.5 mg. of cholesterol) are measured into a similar 10 c.c. cylinder.

To each of the solutions are added 2 c.c. of acetic anhydride and 0.1 c.c. of concentrated sulfuric acid, the solutions mixed by inverting several times, then set away in the dark for fifteen

[1] In order to get an adequate extraction with the small amounts of chloroform used, an excess (3 or 4 c.c.) should be added each time and the mixture allowed to boil down to half its volume or less, before decanting.

[2] It is convenient to make the cholesterol standard in two strengths: (*a*) the stock solution containing 0.2 gm. of cholesterol (Kahlbaum) in 200 c.c. chloroform; and (*b*) the standard solution for use, made by diluting 10 c.c. of the above to 100 c.c. with chloroform. 5 c.c. of this latter solution will contain 0.5 mg.

minutes, after which they are transferred to the cups of the colorimeter (Duboscq) and compared as usual, setting the standard at 15 mm. The cement of the colorimeter cups must, of course, not be soluble in chloroform; plaster of Paris has been found satisfactory, or even ordinary glue if the cups are not used for any other purpose. The error of the above method when carried out with ordinary care is 4 to 5 per cent. If greater accuracy is desired it may be obtained, at the expense of more material and time, by using 50 c.c. of the alcohol-ether extract, evaporating as above, extracting with larger quantities of chloroform, making the extracts to 25 c.c., and taking an aliquot of 5 c.c. for the determination.

CHOLESTEROL

Lichtenthaeler Modification of the Method of Autenrieth and Funk [1]

Principle.—The blood or serum is boiled with strong alkali to saponify the fats. The alkaline solution is extracted with chloroform to separate the cholesterol. After being purified, dried and clarified, the chloroform extract is treated with sulphuric acid and acetic anhydride, and the characteristic blue-green color of the Liebermann-Burchard test for cholesterol is thus obtained. The color is then compared, in a colorimeter, with that produced by a known amount of cholesterol.

Procedure.—With an accurate pipette transfer 2 c.c. of whole blood, serum or corpuscles to a 100 c.c. Erlenmeyer flask provided with a straight tube reflux condenser, and into which has previously been introduced 20 c.c. of a 25 per cent potassium hydroxide solution. Heat on the water-bath, shaking frequently, continuing the digestion until the liquid is colorless or greenish and contains suspended solids derived from certain decomposed substances. This usually requires from three to six hours. Transfer the undiluted mixture to a separatory funnel and add 25 to 30 c.c. of chloroform. Shake vigorously for fifteen minutes and separate. Extract with three additional 25 c.c. portions of chloroform, shaking from five to ten minutes each time. Return the combined chloroform extracts (which are turbid and usually colorless but may be greenish or brown) to the separatory funnel. Wash once or twice by shaking with 15 c.c. portions of distilled water (shaking

[1] Autenrieth and Funk: Münch. Med. Woch., 1913, 60, 1243.

about one minute). Draw off the chloroform layer into a flask or beaker and add 5 to 10 gms. of anhydrous sodium sulphate, bring to boiling on a water-bath or hot plate, filter into a 100 c.c. volumetric flask and dilute to the mark with chloroform. (The washing and treatment with anhydrous sodium sulphate and heat are very important as they bring about the removal of substances which if present render the subsequent comparison of colors very difficult if not quite impossible.) Transfer 10 c.c. of this extract to a small glass-stoppered bottle of about 20 c.c. capacity, add 4 c.c. of pure acetic anhydride, 0.2 c.c. of concentrated sulphuric acid and shake. (If the acetic anhydride has become brownish or yellowish satisfactory results are impossible, due to the development of a brownish-green color. When such discoloration has occurred the acetic anhydride should be redistilled before using. The anhydride should distill at about 136° C. However, the distillate between 134° and 140° C. may be used.) Place in a water-bath or incubator at 35° to 38° C. and allow to stand fifteen minutes in the dark. A blue-green color is developed. At the same time a series of standards are prepared and treated in the same manner. The color of the unknown is compared, in a colorimeter, with that of the most similar of the standards. The average of several closely agreeing readings should be taken. Such readings should be made within a period of twenty to thirty minutes. Four standards are kept, these being prepared by dissolving in 100 c.c. portions of chloroform; (1) 2 mg., (2) 4 mg., (3) 6 mg., (4) 8 mg., respectively of pure cholesterol. (It is convenient to make up a stock solution of pure cholesterol containing 100 mg. in 100 c.c. of pure chloroform, diluting this as required to make up the four standards suggested above.) In preparing the standards for comparison 10 c.c. portions of each of the above standard solutions are taken, placed in small glass-stoppered bottles and treated with acetic anhydride and sulphuric acid as was the unknown. The standards then represent, with the above quantities and dilutions, cholesterol concentrations of: 100 mg., 200 mg., 300 mg., and 400 mg., respectively, in 100 c.c. of the original blood or serum sample used. The 4 mg. standard solution is usually satisfactory, but for blood of very low or high cholesterol content the other standards should be employed.

Calculation.—

$$x = \frac{\text{Standard reading} \times \text{Cholesterol Equivalent of standard}}{\text{Unknown reading}}$$ where x

represents the number of milligrams of cholesterol in 100 c.c. of whole blood, blood serum or corpuscles.

CALCIUM IN BLOOD

Method of Lyman [1]

Principle.—Blood is freed from protein by trichloracetic acid. Calcium is precipitated as the oxalate, dissolved in HCl, and treated with a solution of ammonium stearate. The resulting cloudiness due to formation of calcium soap is compared in a nephelometer with a standard solution of calcium soap.

Procedure.—To draw the blood, a paraffined pipette attached to a hollow needle by a piece of rubber tubing is employed. The potassium oxalate is naturally omitted. Run 5 c.c. of blood into a small flask containing 15 c.c. of trichloracetic acid, 6.5 per cent, while agitating the flask. Mix and let stand for a few minutes. Filter through a folded calcium-free filter paper. Pipette 10 c.c. of the filtrate into an Erlenmeyer flask of about 50 c.c. capacity. Add one drop of methyl orange, 0.1 per cent. Add 2 N ammonium hydrate drop by drop until just yellow. Add nitric acid, 0.05 N, dropwise until pink, and then 1 c.c. more. Add 1 c.c. of oxalic acid, 4 per cent. Add 1 c.c. of sodium acetate, 20 per cent, dropwise. Cool under the water tap until a faint cloud appears. Shake ten minutes or stand over night at room temperature, as convenient. Rinse the stopper with a few drops of ammonium oxalate, 0.5 per cent. Pour into a centrifuge tube and centrifuge. Pipette off supernatant liquor. Rinse the flask with 5 c.c. of ammonium oxalate, 0.5 per cent, pour into centrifuge tube, stir, rinsing with rod with 0.5 per cent ammonium oxalate, and again centrifuge. Pipette off supernatant liquor. Dissolve precipitate in 5 c.c. of 0.1 N nitric acid by means of stirring, and pour into original flask. Agitate a moment to dissolve any precipitate adhering to the walls. Rinse the rod and centrifuge tube with 5 c.c. of water, and pour into the flask.

Into another flask of about 100 c.c. capacity, pipette 20 c.c. of the standard calcium oxalate solution. Pipette 50 c.c. and 25 c.c. respectively of the ammonium stearate reagent into two clean dry beakers. Pour the standard solution into the 50 c.c. of reagent,

[1] Lyman: Jour. Biol. Chem., 1917, 29, 169.

and the unknown into the 25 c.c., and pour back twice. Stopper and let stand ten minutes. Fill both nephelometer tubes with the standard, set the left side at 32 mm., and take a careful reading to be sure that the two sides of the instrument are balanced. Replace the standard on the left with the unknown and read. Care should be taken before reading to remove with a glass rod any bubbles adhering to the walls of the nephelometer tubes.

Calculation.—*If the unknown is set at* 32 *mm. and the standard is read, the reading divided by* 4 *will equal the number of mg. of calcium in* 100 *c.c. of blood.* Note that calcium is calculated as calcium and not as the calcium oxide.

Reagents, etc. *Trichloracetic Acid Solution, 6.5 Per Cent.*—This reagent, as well as all other materials, should be tested for calcium before use.

Filter Paper.—As the filter paper used for common laboratory purposes contains considerable calcium, a paper must be selected for filtering the coagulated blood or milk which has been washed in acid. For this purpose Baker and Adamson's paper "A" washed in hydrochloric and hydrofluoric acids was employed. For filtering the reagents absorbent cotton washed first with hydrochloric acid, 10 per cent, then with water, until the wash water is no longer acid to litmus, and finally dried, may be used.

Indicator, Methyl Orange, 0.1 Per Cent.—Dissolve 0.1 gm. of methyl orange in 10 c.c. of alcohol and make up to 100 c.c. with water.

Nitric Acid, 0.1 N and 0.05 N.—The concentrations need not be exact, provided the same strength of acid is used in all steps of the process, including the making up of the standard. For convenience a stock solution of 2 N may be prepared and the lower concentrations made up from this as needed.

Ammonium Hydrate, 2 N.—This need not be accurate. 13.5 c.c. of ammonium hydrate, sp. gr. 0.9, made up to 100 c.c, with water will serve the purpose.

Oxalic Acid, 4 Per Cent.—As 1 c.c. of this solution is used to precipitate the calcium in only 2.5 c.c. of blood, it will easily be seen that the excess over the theory is very large. Unless such an excess is present, however, precipitation does not begin promptly, owing to the low concentration of calcium oxalate.

Sodium Acetate, 20 Per Cent.—20 gm. of crystallized sodium acetate dissolved in 100 c.c. of water.

Ammonium Oxalate, 0.5 Per Cent.

Ammonium Stearate Reagent.—Dissolve 4 gms. of stearic acid and 0.5 c.c. of oleic acid in 400 c.c. of hot alcohol. Add 20 gms. of ammonium carbonate dissolved in 100 c.c. of hot water and allow the mixture to boil for a few moments. Cool. Add 400 c.c. of alcohol, 100 c.c. of water, and 2 c.c. of ammonium hydroxide (sp. gr. 0.9). Filter. This solution should be as clear as freshly distilled water and perfectly colorless. If well stoppered it keeps indefinitely. Before using for analysis, test as follows: Into two flasks pipette respectively 10 and 5 c.c. of the calcium oxalate standard and to the 5 c.c. add 5 c.c. of nitric acid, 0.05 N. Treat both with 25 c.c. of the ammonium stearate reagent and read on the nephelometer. If they do not read exactly 2 to 1 there is some impurity present in the chemicals used. The alcohol—if, as is usual in laboratories, it has stood in a wooden barrel—will give a yellow coloration with ammonia and will contain suspended particles which reflect light in the nephelometer. It should be redistilled with a little calcium carbonate. Stearic acid may be purified by recrystallizing from boiling alcohol. Ammonium carbonate may be resublimed.

Calcium Oxalate Standard, 10 c.c. to contain 0.2 mg. of Calcium in 0.05 N HNO_3.—Dissolve 72.9 mg. of pure calcium oxalate ($CaC_2O_4+1H_2O$) in 25 c.c. of nitric acid, 2 N, and make up to 1000 c.c. with water. Since the presence of chlorides affects the solubilities of calcium soaps, nitric acid is used as a solvent throughout instead of hydrochloric.

CALCIUM OF THE BLOOD

Method of Marriott and Howland [1]

Principle.—The protein blood serum is destroyed by heating with strong nitric acid. The calcium is then precipitated as calcium oxalate dissolved in hydrochloric acid and determined colorimetrically by means of standard oxalate solutions.

Procedure.—2 c.c. of clear serum are measured into a 50 c.c. conical beaker, 10 c.c. of concentrated nitric acid are added, and the beaker is heated on an electric stove just below the boiling

[1] Marriott and Howland: Jour. Biol. Chem., 1917, 32, 233.

point for two or three hours. The heat is then increased and the acid evaporated down to about 0.5 c.c. Sputtering must be guarded against and the contents must not go completely to dryness. (If charring occurs, more acid must be added and evaporation repeated.) The sides of the beaker are washed down with 2 or 3 c.c. of water and a drop of phenolsulfonephthalein is added as an indicator. Dilute ammonia is added drop by drop until alkaline. (The indicator changes from an eosin pink to yellow and then to reddish purple, the latter color being the alkaline end-point.) The beaker is then replaced on the stove and heated until the excess of ammonia is removed, as shown by a change in color of the indicator and the fact that only a faint odor of ammonia remains. Unless a very great excess of ammonia has been added, two or three minutes at medium heat is usually sufficient. While still hot 1 c.c. of a solution of 1.25 per cent oxalic acid in 0.25 N hydrochloric acid is added drop by drop and with stirring. The beaker is removed from the stove and when cool 0.5 c.c. of sodium acetate solution (20 per cent) is added slowly and with stirring. (This refers to the anhydrous salt. If crystalline sodium acetate is used the solution should be made up to a strength of 35 per cent.) The solution is allowed to stand over night. This method of precipitation, which is essentially that advised by McCrudden, results in a granular precipitate of calcium oxalate, that can be readily filtered without loss.

Filtration is carried out on a 10 c.c. Gooch crucible, the mat being especially prepared as follows: A small disk of filter paper is first placed in the bottom of the crucible, asbestos soup is poured on to make a fairly thick mat, another disk of filter paper is laid on and then a little more asbestos, and finally a suspension of purified barium sulfate. This latter serves to make evident any leaks in the crucible and also to close the pores.

The calcium oxalate precipitate is washed into the crucible and beaker and crucible are washed eight times, each time with approximately 5 c.c. of 1 per cent ammonia (one part concentrated ammonia in 100 of water), then once with 95 per cent alcohol, containing just enough ammonia to be alkaline, and finally once with ether. (Ether which contains acid should be redistilled over sodium hydroxide before using.) The suction is best diminished when the ether is poured on the crucible so as to prevent excessively rapid filtration. After all the ether has passed through

the filter, the suction is increased and after five or ten minutes the mat is quite dry.

The crucible is returned to the beaker and 10 c.c. of 0.2 N hydrochloric acid are run into the crucible and allowed to percolate through. The beaker is covered by a piece of rubber dam held in place by a rubber band or put in a desiccator over water, in order to prevent evaporation. After standing several hours or over night the asbestos is thoroughly stirred up in the acid and the whole suspension transferred to a tube and centrifuged. An aliquot portion of the clear supernatant fluid (usually 6 c.c.) is pipetted off and used for the colorimetric determination.

Colorimetric Comparison.—Small Nessler tubes approximately 120 mm. long and 10 mm. internal diameter are used. They are graduated at 10 c.c. and the bores of the tube should be such that the graduation on each tube should be within 2 mm. of the graduation on any other tube in the set. Round-bottomed tubes may be used, but those with flat bottoms are preferable. Aliquot portions of the oxalate solution are measured into the tubes. Portions of a standard calcium oxalate solution are measured into other tubes. Then to each tube are added 2 c.c. of ferric thiocyanate solution accurately measured with an Ostwald pipette. Each tube is filled to the mark with 0.2 N hydrochloric acid, and the contents are mixed by inverting several times, the ends of the tubes being closed with a clean rubber stopper.

Color comparisons are made by looking lengthwise through the tubes against a dull white background. Readings are first made against standard oxalate solutions differing from each other by 0.5 c.c., interpolating when necessary. Extra standards may then be made up to obtain more exact results.

Calculation.—The calculation is simple, as, for example, 2 c.c. of serum were used, and a 6 c.c. aliquot portion of the oxalate solution was taken. The color in the comparison tube, after adding thiocyanate and diluting to the mark, was found to match that corresponding to 6.3 c.c. of the standard oxalate solution; then

$$6.3 \times .02 \times \tfrac{10}{6} \times \tfrac{100}{2} = 10.5 \text{ mg. per } 100 \text{ c.c. of serum.}$$

It is necessary to run blank determinations on the reagents used and to make allowance for any blank found to be present. Usually a blank amounting to more than 0.1 c.c. of the standard

oxalate solution is not found. On the other hand some samples of nitric acid contain appreciable amounts of calcium.

Preparation of Solutions. *Standard Calcium Oxalate Solution.*—The standard calcium oxalate solution is made by dissolving 0.0630 gm. of pure oxalic acid ($H_2C_2O_4 \cdot 2H_2O$) in water. To this are added 200 c.c. of N hydrochloric acid and sufficient calcium chloride solution to correspond to 20 mg. of calcium. The whole is made up to 1 liter with water. One c.c. of this solution is equivalent to 0.02 mg. of calcium as calcium oxalate.

Ferric Thiocyanate Solution.—The ferric thiocyanate solution is made from two solutions which are mixed one-half hour before use. Solution A is 0.3 per cent ammonium thiocyanate. Solution B is 0.3 per cent ferric chloride, made up from the salt with its contained water of crystallization, adding a few drops of acid, if necessary, to clear the solution. Five c.c. portions of Solutions A and B are mixed and the whole is diluted to 25 c.c. with water. This is the proper dilution for use with ordinary serum, but when smaller amounts of calcium are present the solution should be more dilute; with larger amounts it should be more concentrated.

CALCIUM IN BLOOD

Method of Kramer and Howland [1]

Principle.—The blood serum is ashed. The calcium is precipitated by a procedure which in principle is identical with that of McCrudden. A definite amount of 0.1 N oxalic acid in 0.05 N sulfuric acid equal to about four times the equivalent of calcium present is added. The final volume is made up to 2 c.c. and an aliquot of the filtrate is titrated with 0.01 N potassium permanganate.

Procedure.—1 or 2 c.c. of serum or plasma are measured into a platinum crucible, evaporated to dryness on the water bath, and dehydration is completed in the oven at 110° C. The crucible is then placed in a quartz dish over a Méker or Fisher burner. It is heated gently at first and then more vigorously. The outer dish is then covered with a quartz plate and the heating continued until ashing is complete. The platinum crucible is then removed and the ash dissolved in a small quantity of N HCl. It is

[1] Kramer and Howland: Jour. Biol. Chem., 1920, 43, 35.

again evaporated to dryness and ashed as before. A crystalline material is obtained which is readily soluble in not more than 1 c.c. of 0.1 N H_2SO_4. (About 0.5 c.c. of 0.1 N sulfuric acid is added and solution of the ash facilitated by warming over the steam bath. The solution is then transferred with a small pipette to the Pyrex tube and the process repeated by using two additional portions of 0.25 c.c. of 0.01 N sulfuric acid.) The solution is transferred to a Pyrex tube 100 mm. in length and 10 mm. in diameter calibrated for 1 and 2 c.c. The tube is heated for a few minutes in the water bath and a drop of 0.01 N potassium permanganate is added to prove the absence of any oxidizable material. The pink color should persist for at least one minute. One drop of 0.01 per cent phenolsulfonephthalein is added followed by one drop of concentrated ammonia. The tube is then heated on the water bath to drive off the excess of ammonia. At this point a fluffy precipitate forms. To the tube while hot is added exactly 0.3 c.c. of 0.1 N oxalic acid in 0.05 N sulfuric acid. This is added in three portions with shaking after each 0.1 c.c. The fluffy precipitate dissolves and is replaced by a copious fine crystalline precipitate. The reaction is usually acid at this point. If it is not, sufficient 0.1 N sulfuric acid is added until the color is lemon-yellow which corresponds to a pH of 6.4 to 6.6. Heating is then continued for a few minutes. The tube is then cooled and 0.1 c.c. of a saturated solution of sodium acetate is added and the tube well shaken. The volume is made up to 2.0 c.c. and the tube allowed to stand several hours or over night. The material is then filtered through hardened filter paper. 1 c.c. of the filtrate is measured into a small beaker. To this, 1 c.c. of 20 per cent sulfuric acid (20 c.c. of concentrated acid diluted to 100) is added. (Hydrochloric acid should be avoided as it is oxidized to free chlorine by permanganate.) The beaker is heated on the water bath for a few minutes and then titrated *in good daylight* to a definite pink that persists for at least thirty seconds. For very accurate work the end-point is determined by comparison with an equal volume of water in a beaker of the same size. The amount of permanganate necessary to give the same intensity of color with an equal volume of water is also determined and this volume is subtracted from the titration. A blank determination should also be made on the reagents. For most purposes the subtraction of 0.04 c.c. of 0.01 N permanganate is all that is necessary.

Calculations.—From the permanganate equivalent of 0.3 c.c. of 0.1 N oxalic acid is subtracted twice (since half the total quantity is titrated) the number of c.c. of 0.01 N permanganate used in the final titration, the latter number, however, having been diminished by the amount of permanganate (0.04 c.c.) necessary to give a permanent pink color to water. The result is multiplied by 0.2, since 1 c.c. of the 0.01 N permanganate is equivalent to 0.64 mg. of calcium oxalate or 0.2 mg. of calcium.

Preparation of Reagents.—0.1 *N Oxalic Acid.*—0.1 N sodium oxalate solution (Sörenson) is made up and a potassium permanganate solution standardized against it. This solution then serves to standardize the N solution of oxalic acid. This N solution is quite permanent if kept in a cool place in the dark, and from it 0.1 N oxalic acid in 0.05 sulfuric acid may be prepared whenever needed.

0.01 *N Potassium Permanganate.*—An approximately 0.01 N solution of potassium permanganate is made by diluting a N or 0.1 N solution and the factor determined by titrating against the known 0.1 N oxalic acid solution. Filter before using.

MAGNESIUM IN BLOOD

Method of Marriott and Howland [1]

Principle.—After precipitation of the calcium (see page 160, this Manual) magnesium is precipitated as ammonium magnesium phosphate. This precipitate is dissolved in HCl and estimated colorimetrically against a standard ammonium magnesium phosphate solution.

Procedure.—After precipitation of the calcium oxalate, as previously described, see page 161, this Manual, the contents of the beaker are rinsed into a conical centrifuge tube and centrifuged for about ten minutes. The clear liquid is syphoned off [2] into a

[1] Marriott and Howland: Jour. Biol. Chem., 1917, 32, 233.

[2] Syphoning is accomplished by means of a small glass tube, one end of which is drawn to a short capillary point and bent upwards. This form of tip prevents the sucking up of any precipitate and permits the removal of almost all of the liquid. The syphon tube is passed through a small double-bored rubber stopper, which fits the centrifuge tube. A short glass tube passes through the other hole in the stopper. The syphon is started by blowing into this tube or by the use of an atomizer bulb.

small casserole or a platinum dish and used for magnesium determination. The calcium oxalate remaining in the centrifuge tube is dissolved in 0.5 c.c. of concentrated nitric acid. This is best accomplished by first adding the acid and then 5 or 10 c.c. of water; the tube is then heated by immersion in a beaker of hot water and the contents are stirred by bubbling air through the tube by means of a capillary glass tube reaching to the bottom of the centrifuge tube and connected with a rubber bulb. The contents of the tube are then transferred to the beaker and evaporated to a volume of about 5 c.c.; the calcium is then reprecipitated and determined exactly as described above.

The liquid syphoned off into the dish contains the magnesium [1] present, to this is added 0.5 c.c. of concentrated sulfuric acid, and the liquid is evaporated to dryness on an electric stove. Ashing is completed over a Méker burner. If a small amount of black residue remains, a drop of sulfuric acid is added, after cooling, and the ashing repeated. To the residue is added 0.5 c.c. of concentrated hydrochloric acid and a little water. The dish is warmed and the contents are transferred to a small (25 c.c.) beaker with several washings of hot water. The beaker is put on an electric stove and the contents are evaporated to a volume of about 3 c.c., a drop of phenolsulfonephthalein is added, and then 1 c.c. of ammonium phosphate solution.[2] 1 c.c. of concentrated ammonia is run in with stirring and the beaker allowed to stand over night. Crystals of ammonium magnesium phosphate separate out on the sides and bottom of the beaker.

The contents of the beaker are transferred to a conical 15 c.c. centrifuge tube and centrifuged for a couple of minutes. The liquid is syphoned off in the manner above described and the precipitate in beaker and tube washed with 10 per cent ammonia (one part concentrated ammonia to nine parts of water), the precipitate in the tube being stirred up each time with the capillary blowing tube. It is unnecessary to dislodge the precipitate from

[1] Approximately 0.2 c.c. of liquid remains in the centrifuge tube, and this amount, which is about 2 per cent of the total, may be allowed for in the final calculation, if extreme accuracy is desired.

[2] Ammonium phosphate solution is made as follows: 25 gms. $(NH_4)_2PO_4$ are dissolved in 250 c.c. H_2O. 25 c.c. of concentrated ammonia are added and the mixture is allowed to stand over night. The following day it is filtered, the filtrate is boiled to remove the excess of ammonia, cooled and made up to 250 c.c.

the sides of the beaker. The washing is repeated four times, each time with from 6 to 10 c.c. of ammonia. The precipitate is finally washed once with 95 per cent alcohol made just alkaline with ammonia. This is syphoned off and tube and beaker are dried in an air oven at about 60° C.

After drying, the contents of beaker and tube are dissolved in 10 c.c. of 0.01 N hydrochloric acid and an aliquot portion is pipetted off for colorimetric determination.

The colorimetric comparison is done in the same way as in the calcium determination with the exception that the thiocyanate solution used is diluted to 40 or 50 c.c. instead of to 25, a standard solution of magnesium ammonium phosphate [1] is used instead of one of calcium oxalate, and dilutions in the Nessler tubes are made with 0.01 N hydrochloric acid. The calculation of results is as follows: If 2 c.c. of serum were used and a 6 c.c. aliquot portion of the solution of the precipitate were taken, and the color found to be the same as that of 3.2 c.c. of the standard magnesium solution, then

$$3.2 \times 0.01 \times \tfrac{1}{6}\tfrac{0}{} \times \tfrac{1}{2}\tfrac{0}{0} = 2.66 \text{ mg. magnesium per 100 c.c. of serum.}$$

MAGNESIUM IN BLOOD

Method of Denis [2]

Principle.—Magnesium is determined in the filtrate of blood, or plasma (after removal of calcium by Lyman's Method, see page 158, this Manual), by precipitation of the magnesium as magnesium ammonium phosphate and the nephelometric estimation of the phosphate by means of a strychnine molybdate reagent.

Procedure.—5 c.c. of citrated plasma, serum, or whole blood are measured into 15 c.c. of 6.5 per cent trichloroacetic acid solution, and after shaking the mixture is allowed to stand for at

[1] This solution is made by dissolving 0.102 gm. of air-dried magnesium ammonium phosphate ($MgNH_4PO_4 \cdot 6H_2O$) in 100 c.c. of 0.1 N hydrochloric acid and diluting to 1 liter with water. Of this solution 1 c.c. is equivalent to 0.01 mg. of magnesium. Magnesium ammonium phosphate loses water of crystallization when heated and must therefore be dried at room temperature. Commercial preparations of the salt are generally unreliable; it should be prepared by precipitation of pure solutions.

[2] Denis: Jour. Biol. Chem., 1920, 41, 363.

least thirty minutes, and is then filtered through a dry filter. 10 c.c. of this filtrate (equivalent to 2.5 c.c. of serum) are used for the determination of calcium according to the method of Lyman (see page 158). In this method calcium is precipitated essentially according to the well known technique of McCrudden and the crystals of calcium oxalate are then collected and washed by the help of the centrifuge. For the determination of magnesium the supernatant liquid remaining in the centrifuge tubes is collected by means of a small syphon, as is also the portion of ammonium oxalate solution used as wash liquid; the total amount of fluid collected in this way is placed in a flat-bottomed platinum dish and evaporated to dryness after the addition of 3 c.c. of 10 per cent sulfuric acid; the residue in the dish is then ignited over a free flame until white, an operation which should be complete in two or three minutes. When cool the white residue is dissolved in about 5 c.c. of distilled water, and 10 per cent hydrochloric acid is added drop by drop until the solution of ash is acid to methyl orange. This solution is transferred quantitatively to a 100 c.c. beaker, using distilled water to conclude the operation, and evaporated to a volume of 2 to 3 c.c.; concentrated ammonium hydroxide is then added drop by drop until the solution is alkaline, and finally 0.5 c.c. of 10 per cent ammonium phosphate solution containing 50 c.c. of concentrated ammonium hydroxide per liter. The beaker is covered with a watch-glass and allowed to stand over night. The next day the liquid is poured into a conical centrifuge tube, and the beaker washed with 20 per cent alcohol containing 50 c.c. of concentrated ammonium hydroxide per liter. After centrifuging, the liquid in the tube is removed by means of a small siphon, and the beaker and tube are again washed with about 10 c.c. of the alcohol-ammonia mixture. Three more portions of wash liquid should be used, the precipitate being thoroughly stirred after each addition of fresh liquid.

The precipitation and subsequent washing just described are essentially the process described by Marriott and Howland in their micro method for the determination of mangesium in blood (see page 165, this Manual).

This portion of the procedure is at once the most important and the most unsatisfactory feature of the determination. Both in the method of Marriott and Howland and in the procedure described in this paper, recourse has of necessity been had to a

principle which every chemist will recognize as theoretically incorrect, viz., the determination of magnesium by the measurement of the phosphate combined as ammonium magnesium phosphate, the precipitation of which latter compound must be carried on in the presence of a large excess of phosphate. It is obvious that extremely careful work is essential in the washing of the precipitate in order to avoid contamination with residual traces of ammonium phosphate; furthermore, as is well known, it is possible to overwash an ammonium magnesium phosphate precipitate, so that low results are obtained.

After the removal of the last portion of wash liquid, the tube and beaker are allowed to stand, preferably on a water bath or register until the ammonium has evaporated, and the ammonium magnesium phosphate is then dissolved in 10 c.c. of 0.1 N hydrochloric acid and transferred to a 100 c.c. volumetric flask by means of distilled water. The solution is then made to volume with distilled water, mixed, and the phosphate determined by means of the strychnine molybdate reagent (see Bloor's Method for Phosphoric Acid in Blood, this Manual, page 171).

For the determination of magnesium in normal plasma or serum 25 c.c. of the above solution are usually the most convenient to use, but larger or smaller amounts are sometimes called for.

To the amount of solution taken is added a quantity of distilled water sufficient to bring the volume to 50 c.c., then 25 c.c. of the strychnine molybdate reagent are added. After standing for a period of five minutes the suspension is read against a standard containing 0.01 mg. of magnesium in a volume of 50 c.c. [1] to which have been added 25 c.c. of the strychnine molybdate reagent, and which has been allowed to stand for the same length of time as the unknown.

[1] This standard is prepared from ammonium magnesium phosphate by dissolving 1.02 gms. of the pure salt in 100 c.c. of N hydrochloric acid and diluting to a volume of 1 liter with distilled water; 1 c.c. of this solution is equivalent to 0.10 mg. of magnesium. For the preparation of this dilute standard a portion of the strong solution is diluted to 50 times with 0.1 N hydrochloric acid so that 5 c.c. of the resulting solution will be equivalent to 0.01 mg. of magnesium.

PHOSPHATES OF THE BLOOD

Method of Marriott and Haessler [1]

Principle.—Phosphates are precipitated from serum with magnesia mixture. The precipitate is separated and dissolved in hydrochloric acid. The hydrochloric acid solution of phosphates is then subjected to a colorimetric estimation.

Procedure.—Dilute 1 c.c. of clear serum with about 5 c.c. of water in a small beaker. Add 2 drops of 0.1 N hydrochloric acid and 1 c.c. of magnesia mixture. Then with stirring, run in slowly 2 c.c. of 10 per cent ammonia (one part concentrated ammonia to nine parts of water), and allow to stand over night in a cool place.

The precipitate is filtered off on a small Gooch crucible, the mat being prepared as for the calcium determination. Wash the precipitate and beaker four times, each time with about 5 c.c. of the 10 per cent ammonia, then once with 95 per cent alcohol made just alkaline with ammonia, and finally with 5 c.c. of ether. Suction is kept on for several minutes in order to dry the mat. The crucible is then returned to the beaker and 10 c.c. of 0.01 N hydrochloric acid are run in and allowed to percolate through the crucible. The beaker is covered with a rubber dam or put in a desiccator over water and allowed to stand for a couple of hours or over night. The asbestos is then stirred up in the acid and the suspension transferred to a centrifuge tube and centrifuged. A 6 c.c. aliquot portion of the clear liquid is pipetted off and used for colorimetric determination. This is carried out exactly as for the calcium determination except that the ferric thiocyanate solution is diluted to 40 or 50 c.c. A standard of magnesium ammonium phosphate solution is used in place of calcium oxalate. Each tube is diluted to the mark with 0.01 N hydrochloric acid, and the contents are mixed by inverting several times, the ends of the tubes being closed with a clean rubber stopper.

Calculation.—The calculation of results is as follows: If 1 c.c. of serum were used and a 6 c.c. aliquot portion of the dissolved precipitate taken for colorimetric determination, the color in the Nessler tube corresponding to that resulting from the addition of 0.9 c.c. of the standard phosphate solution, then

$$0.9 \times 0.02 \times \frac{10}{6} \times \frac{100}{1} = 3.0 \text{ mg. phosphorus per 100 gms. of serum.}$$

[1] Marriott and Haessler: Jour. Biol. Chem., 1917, 32, 241.

Preparation of Solutions. *Magnesia Mixture.*—Dissolve 10 gms. of magnesium chloride (sticks) and 5 gms. of ammonium chloride in 250 c.c. of water; add 10 c.c. of concentrated ammonia. Allow to stand over night, filter, neutralize with hydrochloric acid, using phenolsulfonephthalein as an indicator, and make up to 500 c.c. with water.

Standard Magnesium Ammonium Phosphate Solution.—This solution is made by dissolving 0.1584 gm. of air-dried magnesium ammonium phosphate ($MgNH_4PO_4.6H_2O$) in 100 c.c. of 0.1 N hydrochloric acid and diluting to 1 liter with water. One c.c. of this solution is equivalent to 0.02 mg. of phosphorus.

PHOSPHORIC ACID IN BLOOD

Bloor's Method [1]

Principle.—Organic material in the blood is decomposed by treatment with acid. The phosphates are then precipitated as strychnine phosphomolybdate and the amount is measured nephelometrically by comparison with a standard phosphate solution.

Total Phosphates, Whole Blood

Procedure.—3 c.c. of blood are measured with a pipett into a 25 c.c. graduated, glass-stoppered flask, the flask is filled to the mark with water, and the solution well mixed. 1 c.c. of the solution (equivalent to 0.12 c.c. of blood) is measured into a large (200×25 mm.) test-tube, 1.5 c.c. of a mixture of concentrated sulfuric and nitric acids and a few glass beads are added, and the whole is heated with a micro-burner in the hood. The heating is carried out in three stages. In the first stage the mixture is raised to boiling and then the flame turned down until only a slow but constant bubbling takes place. Heating is continued at this rate until red fumes cease to come off. The time required varies with the sample but ordinarily is not more than fifteen minutes. In the second stage of heating the flame is increased until the water is driven off and strong heating with volatilization of a part of the sulfuric acid is continued for eight to ten minutes,

[1] Bloor: Jour. Biol. Chem., 1918, 36, 33.

taking care not to heat so strongly that the tube approaches dryness, in which case loss of phosphoric acid may occur. The sulfuric acid solution should now be clear and colorless. If it is brownish in color, a drop of HNO_3 should be added and the heating continued for one minute. In the third stage the mixture is allowed to cool somewhat (for about two minutes) and then one or more drops of 1 per cent cane sugar solution is added. (The amount added should be enough to produce a deep browning of the *hot* solution and the color should disappear when it is boiled. If too much sugar solution had been added and the brown or yellow color persists after a half minute of boiling, a trace of nitric acid should be added and the boiling continued.) The solution is then boiled until the moisture is gone—about one minute—then cooled and about 10 c.c. of water are added, rinsing down the sides of the tube. The solution in the tube is neutralized by approximate titration with 10 per cent NaOH (from sodium) using one drop of 0.3 per cent phenolphthalein as indicator, noting the amount of alkali added, then made just acid with a drop or two of dilute sulfuric acid (25 per cent). It is then cooled, transferred quantitatively to a 25 c.c. glass-stoppered graduated flask, the tube rinsed several times with water and the washings added to the flask, and the volume made up to the mark with water, and the whole well mixed.

Phosphate Reagent.—*Sodium Molybdate.*—Prepared either according to the direction of Kober and Egerer or, more simply, in the following way. 72 gms. of molybdic acid are mixed with about 300 c.c. of water and neutralized with 40 per cent sodium hydroxide (free from all but traces of phosphates). Pure acid requires the theoretical amount of 100 c.c., impure samples require less. The molybdate, now in clear solution, is boiled for about a half hour, adding water to keep the volume constant and alkali if the solution becomes turbid. About 1 gm. of talcum powder is added and after a further five minutes boiling the solution is filtered and the filter washed once with hot water, adding the washings to the main filtrate. After cooling, the solution, containing approximately 100 gms. of sodium molybdate, is ready for use. Most of the molybdic acid now available contains ammonia, which is volatilized during the boiling and allows some of the acid to precipitate, hence the need of more alkali during the boiling.

Preparation of the Reagent.—Sufficient of the above solution to contain 30 to 35 gms. of sodium molybdate (or this amount of dry sodium molybdate dissolved in a small amount of water) is measured into a precipitating jar or large beaker (2 liters), and 250 c.c. of a mixture of equal parts of concentrated HCl and water are added with stirring. 500 c.c. of water are mixed with the solution and 40 to 50 c.c. of saturated strychnine sulfate solution slowly added with stirring. 200 c.c. more of the dilute acid and 500 c.c. more water are added and, after mixing, the turbid solution is allowed to stand over night or longer if convenient. Next day the precipitate has settled and most of the liquid may be poured off clear. The remainder is filtered through a hardened, phosphorus-free filter. For use in the determination, 25 c.c. of this solution are taken without further additions.

Standard Phosphate Solution.—5 c.c. of the standard acid potassium phosphate solution (containing 0.15 mg. of H_3PO_4) are measured into a 25 c.c. glass-stoppered graduated flask, a drop of phenolphthalein is added, and the amount of alkali used in neutralizing the digestion mixture above run in. The solution is then made just acid with the 25 per cent H_2SO_4, cooled, made up to the mark with water, and well mixed.

Precipitation.—25 c.c. portions of the strychnine molybdate reagent are measured into each of two 50 c.c. glass-stoppered, graduated flasks. 5 c.c. of the standard solution are run into one of the flasks, which is kept gently rotating during the addition, and 5 c.c. of the test solution similarly added to the other. When the solutions are well mixed they are allowed to stand at least three minutes, then filled to the mark, and mixed by inverting several times, after which they are ready to be compared in the nephelometer.

Reading.—The nephelometer tubes are filled with the solutions to the same height and to the point at which, when the tubes are in position in the nephelometer, the meniscus is just out of reach of the light. The jacket on the standard tube is set at a convenient point and readings are made as usual.

Plasma

0.5 c.c. of plasma is measured with a 0.5 c.c. Ostwald pipette into a large test-tube, glass beads and 1.5 c.c. of the sulfuric-

nitric acid mixture are added, and the solution is treated as with whole blood. If a 0.5 c.c. pipette is not available, the plasma may be diluted with an equal volume of water and 1 c.c. taken.

Corpuscles

Plasma and corpuscles are separated by centrifugation for ten minutes at about 4000 R.P.M., the plasma is removed as completely as possible, the corpuscles are washed once by shaking with a volume of 0.9 per cent salt solution equal to the volume of plasma, then centrifuged at once in the same way as before. If this operation is performed quickly, the small remaining amount of plasma is washed out without significant change in the phosphate content of the corpuscles (by dialysis). 0.08 c.c. of corpuscles gives a total phosphate content of about the strength of the standard used. To obtain this amount, 1 c.c. of the corpuscles is measured with an Ostwald pipette into a 25 c.c. glass-stoppered, calibrated flask, the pipette rinsed clean with water, the rinsings are added to the flask, and the mixture is made up to volume with water, and mixed. 2 c.c. of this dilution (0.08 c.c. of corpuscles) are measured into a large test-tube, then treated as in the case of whole blood.

Lipoid Phosphoric Acid ("Lecithin")

Whole Blood

Three c.c. of well mixed whole blood is measured into a 100 c.c. flask containing about 75 c.c. of a mixture of 3 parts alcohol and 1 part ether (both redistilled). The blood is made to enter in a slow stream of drops and the liquid in the flask kept rotating rather rapidly so as to prevent the formation of large aggregates of precipitate which are difficult to extract. The flask and contents are then immersed in boiling water with frequent and strong rotation (to prevent superheating) until the liquid begins to boil, cooled to room temperature, made up to volume, mixed, and filtered. For the determination 10 c.c. (=0.3 c.c. of blood) are measured into one of the large test-tubes, glass beads added, and the whole is evaporated to dryness in a boiling water bath. The tube should be shaken frequently until boiling begins, after which the solution will proceed quietly to dryness.

It should be left in the bath a few minutes after it is apparently dry to remove traces of alcohol which would interfere with the subsequent oxidation. 1.5 c.c. of the sulfuric-nitric acid mixture are added, distributed by shaking to the material on the sides of the tube, and the mixture is digested in the same way as directed for total phosphates.

Plasma

Two c.c. of plasma are measured into 35 to 40 c.c. of alcohol-ether in a 50 c.c. flask as described above and the whole process is carried out as for whole blood. For the determination 15 c.c. is required (0.6 c.c. of plasma).

Corpuscles

Corpuscles are hemolyzed by dilution with an equal volume of warm water and allowed to stand for ten minutes, then 3 c.c. of the dilution are measured into 35 to 50 c.c. of alcohol-ether in a 50 c.c. flask and treated as with whole blood. There is a tendency for the corpuscular precipitate to mass together when it settles out and the flask should be well shaken occasionally during a half hour after which the extraction may be proceeded with as before. For the determination 10 c.c. of the extract (0.3 c.c. of corpuscles) are used.

Acid-Soluble Phosphoric Acid—Inorganic and Other Forms

Whole Blood

The blood is first laked by the addition of an exactly equal volume of warm (40° C.) water and allowed to stand for about ten minutes with occasional shaking. Of this mixture 5 c.c. are measured with a pipette into 15 c.c. of acid ammonium sulfate (see acid ammonium sulfate in the notes below, page 178) in a 25 c.c. glass-stoppered graduated flask. The blood is added slowly and the liquid in the flask kept rotating during the addition, after which the flask is filled to the mark with the ammonium sulfate, well mixed and let stand with occasional shaking for at least ten minutes. The liquid is then filtered through a phosphate-free filter. The filtrate is clear, colorless, and free from protein. In this filtrate determinations are made of acid-soluble, inorganic, and, by difference, other forms of phosphoric acid.

Inorganic Phosphoric Acid.—10 c.c. of the filtrate (=1 c.c. of original blood) are measured into a 25 c.c. glass-stoppered flask, made to the mark with water, and mixed. The standard is prepared by measuring 3 c.c. of the standard phosphate solution (0.09 mg. of H_3PO_4) into a similar flask, adding 8 c.c. of the acid ammonium sulfate (to balance the salt content of the test solution), filling to the mark with water, and mixing. Determinations are made with 5 c.c. of this dilution as in total phosphates.

Acid-Soluble Phosphoric Acid.—2 c.c. of the filtrate (0.2 c.c. of blood) are measured into one of the large test-tubes, glass beads and 1.5 c.c. of the sulfuric-nitric acid mixture added, and the whole is digested with heat in the same way as for total phosphates, except that since the amount of organic material is very small, the first stage of heating may be passed over quickly. The neutralization and subsequent treatment are the same as for total phosphates. For a standard solution measure 3 c.c. of the standard phosphate solution (0.09 c.c. of H_3PO_4) into a 25 c.c. glass-stoppered flask, add about 1.5 c.c. of the acid ammonium sulfate and the amount of alkali used in neutralizing the test solution. Neutralize with acid as usual, then make to the mark and mix. Subsequent treatment is as with total phosphates.

Other Forms of Phosphoric Acid.—Obtained by subtracting inorganic from acid-soluble phosphoric acid.

Plasma

Three c.c. of plasma are run slowly into 20 c.c. of the acid ammonium sulfate solution in a 25 c.c. glass-stoppered graduated flask, the volume is made to the mark with water, the whole mixed, and let stand with occasional shaking for at least ten minutes. It is then filtered. After the filter has drained, it should be folded in the funnel and pressed out with a clean stirring rod to get as much filtrate as possible. The filtrate is clear and colorless and contains no detectable protein. With this filtrate determinations are made of inorganic and acid-soluble and other forms as before.

Inorganic.—(*a*) 10 c.c. of the filtrate (=1.2 c.c. of plasma) are measured into one of the 25 c.c. flasks and made to the mark with water. A standard is prepared by adding to another flask 3 c.c. of the standard phosphate (0.09 mg. of H_3PO_4) and acid ammonium sulfate equal to that present in the test solution (8

c.c. is a sufficiently close approximation). The flask is filled to the mark with water, and precipitations and readings are made as usual. The determination should be made promptly after filtering. (*b*) Where many determinations are to be made it is advisable to make a special standard—when determinations may be made more simply as follows:

Standard.—Made by measuring 2 c.c. of the strong stock standard phosphate solution (=1.2 mg. of H_3PO_4) into a 100 c.c. flask, adding 80 c.c. of the acid ammonium sulfate, and making up to 100 c.c. with water. Of this standard 5 c.c. contains 0.06 mg. of H_3PO_4. For the determination 5 c.c. of the filtrate (=0.6 c.c. of plasma) are measured directly into 25 c.c. of the strychnine molybdate reagent in one 50 c.c. graduated flask, 5 c.c. of the standard solution into another, and after mixing and standing three minutes the flasks are filled to the mark, the solutions mixed, and determinations made.

Acid-Soluble.—10 c.c. of the filtrate are measured into one of the large test-tubes, glass beads and 1.5 c.c. of sulfuric-nitric acid mixture are added, and the mixture is digested. The digestion presents some difficulties because of the large amount of ammonium sulfate present. The first stage is passed over quickly, then in the second stage of heating when the mixture thickens and begins to foam, the heat is moderated and so continued until foaming ceases and the salt fuses to a small volume in the tube. Heating is then carried on for about ten minutes at a rate just sufficient to prevent loss of ammonium sulfate from the tube by volatilization. The tubes are then cooled, treated with one drop of 0.3 per cent cane sugar solution, and reheated in the regular way. After dissolving in water the solution is titrated with alkali, noting the amount used. The standard contains 3 c.c. of the standard phosphate solution, 8 c.c. of the acid ammonium sulfate, and the amount of alkali used to neutralize the test solution. The mixture is neutralized and the phosphoric acid determinations are carried out in the regular way.

Corpuscles

Five c.c. of corpuscles are measured with a pipette into a 10 c.c. glass-stoppered graduated flask and the pipette is rinsed clean with small portions of warm water. The rinsings are

added to the flask, the whole is made to volume, mixed, and let stand with occasional shaking for at least ten minutes to allow laking of the corpuscles. 5 c.c. of this dilution are run slowly, with shaking, into 15 to 18 c.c. of the acid ammonium sulfate in a 25 c.c. flask, the volume is made to 25 c.c. with the ammonium sulfate solution, and the whole well mixed. Of this mixture 10 c.c. are measured out and used for the determination of acid-soluble phosphoric acid as directed below. The remainder is allowed to stand at least ten minutes, then filtered, and determinations are made of inorganic phosphates.

Inorganic.—5 c.c. of the filtrate (=0.5 c.c. of corpuscles) are used for the determination which is carried out as in method (*b*) for plasma, using the same standard. Determinations should be made at once after filtering since in certain cases and particularly in warm weather the values have been found to increase on standing, probably at the expense of the unknown acid-soluble phosphoric acid compound.

Acid-Soluble.—10 c.c. of the ammonium sulfate-corpuscle mixture are measured into a small flask and immersed for two minutes in boiling water. After cooling it is poured into a 25 c.c. graduated flask, the small flask is rinsed with 10 c.c. of the acid ammonium sulfate, and the rinsings are added to the main portion in the 25 c.c. flask. The volume is made up to the mark with water, mixed, and filtered. For the determination 2 c.c. of the filtrate (0.08 c.c. of corpuscles) are measured into one of the large test-tubes, glass beads and 1.5 c.c. of the sulfuric-nitric acid mixture are added, the whole is digested, and determinations are made as with total phosphates, the small amount of ammonium sulfate present not interfering. For the standard, 5 c.c. of the standard phosphate (0.15 mg. of H_3PO_4) are used and to it are added 1.5 c.c. of the ammonium sulfate solution; then it is treated with alkali, neutralized, etc., as in the regular determinations.

Notes on the Methods

Solutions Required for these Determinations.—1. *The strychnine molybdate* reagent as described.

2. *Acid ammonium sulfate.* Saturated ammonium sulfate, free from all but traces of phosphate, to which have been added 15 c.c. of glacial acetic acid per liter.

3. *Standard phosphate.* (*a*) Stock standard, containing in 100 c.c. 0.0834 gm. of pure acid potassium phosphate. (*b*) Standard for use: dilute 25 c.c. of the above to 500 c.c. Each 5 c.c. of this standard contains 0.15 mg. of H_3PO_4. The dilute standard has been found to deteriorate in hot weather and should be made up at least once a month.

4. *Sodium hydroxide* (from sodium) 10 to 20 per cent.

5. *Concentrated sulfuric and nitric acids free from all but traces of phosphates* (see below).

6. *Dilute sulfuric acid.* One part of the concentrated acid and 3 parts water.

Blanks run with the acids, alkali, and ammonium sulfate should show only a slight cloudiness on standing for one hour with the reagent. The ordinary highest grade reagents obtainable up to the present time have been found satisfactory with the exception of the sodium hydroxide which should be made from the metal. If the distilled water is stored in metal tanks it may contain lead which is itself a fairly good precipitant for phosphates. The water should therefore be stored in glass.

Because of the minute amounts of phosphoric acid determined the greatest care should be used to avoid contamination, and free use should be made of good distilled water. For the same reason glass-stoppered measuring flasks should be used throughout.

Reagents.—With impure samples of molybdic acid a highly colored solution is often obtained which is difficult to use. It has been found that if the colored solution is exposed to sunlight for a time the color fades and, if the reagent is then taken out of the direct light, does not reappear. (If placed in the *dark*, the color reappears to some extent, and, if exposed to sunlight too long, the reddish purple color changes to a deep yellow which is almost as difficult to work with as the original color.) If pure molybdic acid is used the reagent is colorless but with the above precautions it has been found possible to use acid of varying degrees of purity.

Methods.—The quantities of blood taken are based on the average values for normal human blood and variations in amount of blood or strength of standard may be necessary in special cases.

The tubes with the beads should be given a preliminary "steaming out" by boiling concentrated sulfuric acid in them

for at least half an hour. Four beads about 3 mm. in diameter have been found the most satisfactory number for each tube in the determination. With use they become etched by the strong acid and are then more effective in promoting even boiling than when new.

If the heating of the blood with the acid mixture is hastened too much, the nitric acid may be driven off before oxidation is complete—as shown by a darkening of the solution. If this occurs one or two drops of nitric acid are added, and the heating is continued.

The use of the cane sugar is necessary because of the formation of a varying amount of some compound of phosphoric acid (probably a nitric acid compound) which does not precipitate with the reagent and which is destroyed by organic material added. In the determination of lipoid phosphoric acid the sugar appears to be less necessary, but in determinations of total phosphates low values are invariably obtained unless it is used, and it is safest therefore to use it as a routine procedure in all determinations.

During the boiling of a H_2SO_4—water mixture, first the excess of water is boiled off, then the water vapor is succeeded by a dense white cloud of mixed water and sulfuric acid vapor, and finally by the much thinner vapor of the sulfuric acid alone. In this final stage a clear zone appears between the liquid and the vapors above. The appearance of the clear zone indicates that the heat is strong enough.

Accurate nephelometric measurements are possible only when the size of the particles of the suspension is approximately the same in both standard and test solutions. This similarity is secured by adjustments of conditions as regards salt content, temperature, etc., which are made as nearly as possible the same in both solutions.

Three minutes is the minimum time for the precipitation to come to the point where it may be measured. The length of time that the solutions may stand and still give accurate readings has not been determined, but in solutions no more than 25 per cent apart no significant change has been found in twenty minutes.

The jackets of the nephelometer should fit snugly at the top so that the light is cut off sharply at that point and there should be the minimum of play in either tubes or jackets, otherwise consistent readings are not obtained. It is rarely possible to get

tubes or fittings sufficiently alike so that when both tubes are filled with the same solution and the standard set at a given point say 25 mm., the test solution will read 25 mm. Kober's suggestion has always been followed in this connection; i.e., set the test solution at the required point (say 25 mm.) and adjust the standard until the two fields are the same. Then set the standard at that point and call it 25 mm. The correctness of the position should be tested frequently.

The Richard's type of nephelometer (with moving jackets), made by adapting a Duboscq colorimeter, is believed to be preferable to the plunger type, because no foreign body with its possible sources of contamination comes in contact with the solutions. The danger will be realized when attention is directed to the fact that in ordinary determinations the amounts measured are about 0.03 mg. of H_3PO_4 in 50 c.c. of solution.

Corrections.—The readings obtained in the nephelometer are not exactly proportional to the amounts of phosphoric acid in the solutions. Strong solutions appear stronger and weak solutions weaker than they should. Different schemes for correction of the readings have been suggested, none of which has been found entirely satisfactory and for exact work the necessity for correction is avoided by adjusting the strength of the standard until the correction falls within the limit of error of the method. Solutions up to about 25 per cent stronger or weaker than the standard may be compared without correction. Solutions from 25 to 50 per cent above or below the standard may be read with correction with an accuracy of 3 to 5 per cent of the theoretical value. It is not thought advisable to attempt comparison of solutions varying more than 50 per cent from the standard. The standard phosphate solution contains 0.03 mg. of H_3PO_4 per c.c. and if the regular 5 c.c. make a standard more than 50 per cent above or below the test solutions, a fresh standard should be made with 2 to 10 c.c. of the standard, which will give solutions falling within the ordinary range of blood phosphates. Adjustment of the salt content must of course be made as with the ordinary standard.

In the determination of lipoid-phosphoric acid it makes no difference whether the second part of the extraction—the heating—is done at once or after some time. Ordinarily it has been found convenient to keep the flasks in a cool, dark place for a week or more until a suitable number have accumulated before finish-

ing the process. The extracts kept cool in the dark and tightly stoppered will remain unchanged for two or three months. They may also be used for determination of other blood lipoids as well as "lecithin."

Alcohol-ether has been found to make an exact separation of the lipoid from the acid-soluble forms of phosphoric acid combination in blood. Tests have been made for water-soluble phosphoric acid in these extracts. At no time was there found more than a trace of phosphoric acid—which may have originated from the lecithin—even in cases of severe nephritis where the inorganic phosphate was known to be much above the normal value. As further confirmation of the completeness of the separation, the sum of acid-soluble and lipoid phosphoric acid has been found in both plasma and corpuscles to be very nearly equal (probably within the limits of error of the various determinations) to the total phosphates. Hence if one is known the other may be determined by difference with a good degree of accuracy.

Because of the tendency of the unknown phosphoric acid constituent of the acid ammonium sulfate extract (especially that from the corpuscles) to decompose, yielding phosphoric acid, the determinations of inorganic phosphate should be carried out at once after filtering.

Cold (room-temperature) extraction with acid ammonium sulfate for at least ten minutes was found to give complete extraction of both inorganic and unknown acid-soluble phosphoric acid in whole blood and plasma but not of the unknown in the corpuscles. For this reason heating for two minutes was necessary to recover all of this substance from the corpuscles. Whether heating increased the amount of inorganic phosphoric acid extracted from corpuscles could not be determined because heating decomposes the unknown, yielding phosphoric acid. For that reason inorganic phosphates could be determined only in the extract made in the cold. Since, however, cold treatment gave complete extraction of inorganic from plasma and since added phosphate was completely recovered from corpuscles by the cold extraction, it is believed that it is adequate for the determination of inorganic phosphates in the corpuscles.

To avoid a multiplicity of standards, directions are given for the use in most cases of a single standard. In case a single determination is to be made on a large number of samples it is better

to make a special standard as in method (*b*) for inorganic phosphates in plasma.

SODIUM IN BLOOD

Method of Kramer [1]

Principle.—After ashing blood, serum or plasma, sodium is precipitated by use of potassium pyroantimonate as $Na_2H_2Sb_2O_7 . 6H_2O$. The precipitate is collected in a crucible, dried and weighed.

Procedure.—The ash of 1 or 2 c.c. of blood, serum, or plasma obtained as described for the potassium method (see page 194) is dissolved in water, in a platinum dish, using 0.5 c.c. of water for each c.c. of serum, plasma, or blood. Solution may be aided by the addition of a drop or two of N hydrochloric acid. The solution is then made slightly alkaline with freshly prepared 10 per cent KOH solution. Fifteen c.c. of the reagent and one-fifth of the entire volume of absolute alcohol [2] are then added. Precipitation occurs at once. The mixture is stirred, allowed to stand for at least two hours (preferably over night), then transferred to the wet pad of a weighed Gooch crucible as in the potassium method (see page 194). It is then washed four or five times with 3 c.c. portions of 30 per cent alcohol, dried at 110° C., cooled in a desiccator, and weighed. 1 mg. of sodium yields 11.08 mg. of precipitate. All reagents should be tested for the presence of sodium and ammonium salts, especially the potassium hydroxide. A blank determination should be done and the result subtracted from the Na determination in the sample. Most of the laboratory reagents will give a slight precipitate with the potassium pyroantimonate reagent.

Preparation of the Reagent. — *Potassium pyroantimonate* (J. T. Baker, c.p. analyzed chemicals), 2 gms. of the powder, is added to 100 c.c. of boiling water in a 350 c.c. Pyrex flask and heating continued until no more dissolves. It is then cooled rapidly under the tap and 3 c.c. of 10 per cent KOH are added, and the solution is stirred and filtered. The clear filtrate constitutes the reagent. Although one may be able to precipitate sodium

[1] Kramer: Jour. Biol. Chem., 1920, 41, 271.

[2] The addition of too much alcohol will precipitate some of the reagent, which is itself not very soluble and hence the results will be too high.

with a reagent that has been kept in the ice box for two months, nevertheless, it is always best to prepare the solution fresh each time. The potassium antimonate need be weighed only roughly and the rest of the preparation takes but a few minutes. 10 per cent KOH should preferably be free of both sodium and ammonium salts. Alcohol-washed KOH contains relatively little of these. The exact content, if any is present, should, of course, be determined and a correction made for the amount used. This solution should also be made fresh or kept so that it will neither absorb ammonia nor dissolve sodium.

SODIUM IN BLOOD

Method of Doisy and Bell [1]

Principle.—Blood is either ashed (wet) or freed from protein by precipitation. The sodium in the resulting solution is precipitated as sodium cesium nitrite. The sodium may be estimated gravimetrically, volumetrically or colorimetrically.

Procedure.—1 c.c. of whole blood, plasma, or urine is transferred to a pointed Pyrex tube.[2] A few drops of H_2SO_4 (concentrated) and 5 c.c. of HNO_3 (concentrated) are added. A low flame which keeps the liquid boiling gently is used. The digestion is continued in the usual manner until the liquid is colorless. Urine is completely oxidized in about eight minutes but the blood generally takes three-quarters of an hour.

As both iron salts and any appreciable amount of phosphates interfere with this method of determination of sodium, they must be removed from the blood digest.

The digest of the whole blood is quantitatively transferred to a 25 c.c. volumetric flask with about 20 c.c. of water. One drop of methyl orange and 5 to 6 drops of 4 per cent bismuth nitrate are added. A strong solution of potassium carbonate (free from

[1] Doisy and Bell: Jour. Biol. Chem., 1921, 45, 313.

[2] These tubes are reclaimed from non-protein nitrogen determinations. After a tube has been rendered unserviceable by the phosphoric acid it is heated in an oxygen-gas flame and drawn out to a point. The tubes with small tips and thin walls stand heating best. The pointed tip provides a constant stream of bubbles which promote even boiling. No boiling stones are necessary.

sodium) is added dropwise with shaking until the indicator changes color. The flask is made up to volume, mixed, and the solution transferred to a centrifuge tube. Centrifuging at moderate speed throws down the insoluble phosphates and iron salts.

Twenty c.c. of the supernatant liquid are pipetted into a 50 c.c. Erlenmeyer flask. This solution is evaporated [1] on the hot plate to 2 to 3 c.c. and rendered just acid with HNO_3. An excess of 0.5 c.c. of 2 N HNO_3 is added and the precipitation carried out as described.

If deproteinization is preferred to the process of ashing the following will be found helpful. Five c.c. of whole blood or plasma are transferred to a 50 c.c. flask containing 35 c.c. of water, and 5 c.c. of trichloroacetic acid (20 per cent) are added. The contents of the flask are diluted to the mark, mixed, allowed to stand about thirty minutes, and filtered through a dry paper. Ten c.c. of filtrate (equivalent to 1 c.c. of blood) are pipetted into a 50 c.c. Erlenmeyer flask and 1 drop of concentrated nitric acid is added. The flask, closed with a trap, is heated on a piece of asbestos on a hot plate until brown fumes from the acid are evolved. It is removed, cooled, and the trap washed off with a few drops of water. Precipitation is then carried out as described below.

Precipitation.—The solution is cooled to 10 to 12° C. and 3 c.c. of reagent are added for each milligram of sodium expected. The flask is stoppered with a two-hole rubber stopper bearing two short glass tubes bent at a right angle. One is fitted with a short rubber tube with a glass plug, the other with a Bunsen valve and plug. Illuminating gas freed from H_2S is passed into the flask for a few seconds and the plugs are replaced. The flask is put in the cold room at 1° C. A yellow crystalline precipitate begins to form in a few minutes. Precipitation is complete in twenty-four hours, whereas at room temperature forty-eight hours are required. A scum is much more likely to form before the precipitation is complete at the higher temperature.

The precipitate is rapidly filtered on a Gooch crucible which has previously been dried and weighed. Washing with the ice-cold 50 per cent acetone which is saturated with sodium cesium

[1] The authors advise the use of a trap in the mouth of the flask to prevent loss by bumping. This is made by blowing a bulb on the closed end of a small soft glass test-tube. A hole is then blown in the side of the bulb which is then cut off from the tube.

bismuth nitrite is quickly carried out. Speed during the filtration and washing is essential for good results. Ten c.c. of the 50 per cent acetone are used; 2 c.c are blown from a miniature wash bottle (made from a 10 c.c. graduated cylinder) into the precipitation flask. The suction is stoppered and the liquid poured onto the mat. This is repeated four times. Ten c.c. of pure acetone are used to complete the transfer of the precipitate to the Gooch. If the volumetric or colorimetric procedure is used, complete transference of the precipitate is not necessary.

The Gooch is dried in an air bath at 100° C. until a constant weight is obtained.

Weight of precipitate $\times 0.03675$ = Weight of sodium in solution.

Volumetric Procedure.—The Gooch crucible and contents are placed in a tall 200 c.c. beaker. A large excess of standard permanganate (at least twice the amount necessary for oxidation) and enough water to cover the crucible are added. The precipitate is stirred loose from the crucible and asbestos. Ten c.c. of 1:1 sulfuric acid are poured in while the liquid is being stirred. After a few minutes the solution is heated to 75°C., an excess of standard oxalic acid added, and the titration finished with permanganate. A blank must be run on the reagents under similar conditions.

C.c. $KMnO_4 \times$ normal factor $\times 8$ = Mg. O used
Mg. $O \times 7.82$ = Mg. precipitate
Mg. precipitate $\times 0.03675$ = Mg. sodium.

Colorimetric Procedure.—For those who prefer a colorimetric method a suitable technique follows. The precipitate is completely transferred to a beaker and 10 c.c. of the alkaline tartrate are added. Upon warming, the salt rapidly dissolves. The solution is quantitatively transferred to a 100 c.c. volumetric flask, cooled, made up to volume, and mixed. A further dilution is made so that a volume containing approximately 0.01 mg. of N can be taken for colorimetric comparison.

The standard and unknown in 100 c.c. volumetric flasks are diluted to about 90 c.c. Two c.c. of the sulfanilic acid and naphthylamine solutions are added to each. The flasks are made up to volume, mixed, and allowed to stand twenty minutes for the full color development. There is a very wide range of propor-

tionality of color intensity to the amount of nitrite present. The colors are very stable.

The calculation is simple.

$$\frac{20\times0.01}{\text{Unknown reading}}=\text{mg. N in sample used}$$

Suppose the sample was 1 c.c. of a dilution of 1:1,000 then

$$\text{Mg. N in sample}\times1{,}000=\text{mg. N in precipitate}$$

$$\frac{9CsNO_2.6NaNO_2.5Bi(NO_2)_3}{30\ N}=\frac{3753.6}{420.3}=8.93$$

$$\text{Mg. N}\times8.93=\text{mg. precipitate}$$

$$\text{Mg. precipitate}\times0.0367=\text{mg. sodium in sample}$$

The authors prefer the volumetric method to either the gravimetric or colorimetric on account of its greater speed and accuracy. Possible contamination of the precipitate with either bismuth subnitrate or potassium nitrate renders the gravimetric values doubtful. The colorimetric procedure is open to the usual errors of such methods. The red color is very bright and comparison is rather difficult.

Reagents

1. *Bismuth Cesium Nitrite Solution.*—30 gms. of sodium-free potassium nitrite [1] are dissolved in about 60 c.c. of pure

[1] Pure potassium nitrite has been a source of considerable difficulty which has finally been overcome. The potassium salts on the market generally certain large quantities of sodium. Since the nitrite cannot be purified by recrystallization, the only recourse is to make it. Various samples of carbonate have been examined and it has been found that both Merck's Blue Label and Eastman's are substantially free from sodium. As an emergency procedure sodium-free potassium carbonate may be made by recrystallization of the oxalate. It is dried and ignited in a platinum dish.

The pure nitrite is made by passing nitrous fumes into a 25 per cent solution of sodium-free potassium carbonate. Nitric acid (sp. gr. 1.2) is dropped from a separatory funnel into a flask containing arsenious oxide. A delivery tube carries the fumes into the carbonate. The reaction is complete when the solution in the receiving flask gives off many fine bubbles of carbon dioxide on shaking. Nitrite determinations are run at intervals to ascertain whether the reaction is running perfectly.

water. A solution containing 3 gms. of bismuth nitrate is added. (It is well to keep on hand a 60 per cent solution of the crystallized salt in 2 N HNO_3.) If a precipitate forms (due to excessive alkalinity of the KNO_2), dilute nitric acid is added carefully until it redissolves. A strong solution containing 1.6 gm. of $CsNO_3$ and 1 c.c. of 2 N HNO_3 is added. The solution is diluted to 100 c.c. and dilute nitric acid is used to remove any turbidity which may form. At this stage the reagent should be a clear orange-yellow. If sodium salts were present in any of the chemicals as impurity, the insoluble precipitate which has formed at the end of twenty-four hours is filtered off. The reagent is kept under illuminating gas in the cold room.

2. *Acetone.*—Redistilled and kept ready for use at 1° C.

3. *A 50 per cent solution of acetone* saturated at 1° C. with sodium cesium bismuth nitrite.

For Volumetric Procedure.—1. *Permanganate 0.1 N or 0.05 N.*

2. *Oxalic acid, 0.1 N or 0.05 N.*

3. *H_2SO_4 concentrated acid diluted with equal volume of water.*

For Colorimetric Procedure.—1. *Alkaline tartrate.* Equal volumes of KOH (10 per cent) and tartaric acid (10 per cent) are mixed.

2. *Sulfanilic acid,* 0.8 per cent in 5 N acetic acid.

3. *α-Naphthylamine,* 0.5 per cent in 5 N acetic acid.

4. *Nitrite standard.* Made by recrystallizing $AgNO_2$ from hot water until free from nitrate. Add NaCl equivalent to the $AgNO_2$ and filter off silver chloride. Determine nitrite nitrogen by Devarda's method and dilute so that 5 c.c. = 0.01 mg. N.

Method of Devarda for Nitrite Nitrogen [1]

An amount of this solution which is equivalent to 0.5 gm. nitrite is placed in an Erlenmeyer flask of 600 to 800 c.c. capacity to which are added 60 c.c. water, 5 c.c. alcohol and 50 c.c. of KOH of sp. gr. 1.3. To this solution 2.5 gms. of a powdered amalgam formed from 45 parts of aluminum, 50 parts copper and 5 parts zinc are added; the flask is immediately connected with a distilling apparatus and the resulting ammonia caught in a standard acid.

[1] Devarda: Z. anal. Chem., 1894, 33, 113.

DIRECT DETERMINATION OF SODIUM IN SERUM

Method of Kramer and Tisdall[1]

Principle.—Serum is treated directly with the potassium pyroantimonate reagent and alcohol. The precipitate is collected in a weighed Gooch crucible, dried, and weighed.

Procedure.—Two c.c. of serum are transferred to a platinum dish. To these are added 10 c.c. of the potassium pyroantimonate reagent followed by 3 c.c. of 95 per cent alcohol. The alcohol should be added drop by drop and the specimen stirred with a rubber-tipped rod. After standing forty-five minutes, the precipitate is transferred to a weighed Gooch crucible and washed with 8 to 12 c.c. of 30 per cent alcohol. The crucible is dried at 110° C. for one hour,[2] cooled in a desiccator for thirty minutes and weighed. The weight of the precipitate divided by 11.08 equals the number of mg. of sodium present in the sample.

Preparation of the Potassium Pyroantimonate Reagent

Five hundred c.c. of distilled water are heated to boiling in a Pyrex flask and approximately 10 gms. of potassium pyroantimonate (J. T. Baker) are added. The boiling is continued from three to five minutes, the flask immediately cooled under running water, and when the contents are cold 15 c.c. of 10 per cent KOH (alcohol-washed) are added. The reagent is then filtered through ash-free filter paper into a paraffined bottle. Frequently some of the undissolved potassium pyroantimonate will pass through even the best filter paper. If the reagent is allowed to stand twenty-four hours after filtering, all the undissolved potassium pyroantimonate will settle to the bottom. The supernatant fluid is then clear and may be used as long as it remains so. The reagent keeps perfectly well at room temperature for at least one month. 10 c.c. of this reagent will precipitate 11 mg. of sodium. The 10 per cent KOH should also be kept in a paraffined bottle.

Before the reagent is used for the first time, it should be tested for the presence of sodium and also the fact ascertained that none

[1] Kramer and Tisdall: Jour. Biol. Chem., 1921, 46, 467.

[2] The crucible is placed in the oven the temperature of which is *gradually* raised to 110° C.

of the potassium pyroantimonate is precipitated by the addition of alcohol in the proportion used in the method. This is accomplished by adding to 10 c.c. of the reagent in a platinum dish 2 c.c. of distilled water and 3 c.c. of 95 per cent alcohol.

Details of the Method

Serum.—The serum may be separated from the clot any time within twenty-four hours after collection of the sample, as it has been found that the sodium content of normal serum is not changed during this time by contact with the clot.

Platinum. The platinum dishes must be scrupulously clean, otherwise the precipitate has a tendency to adhere to the sides. The dishes are cleaned with fine sand, then rinsed with distilled water.

Addition of Reagent and Alcohol.—No special precautions are necessary for the addition of the reagent. The 95 per cent alcohol has to be added drop by drop while the mixture is stirred with a rubber-tipped rod. Redistilled alcohol should be used.

Precipitation. — Precipitation is complete from thirty to forty-five minutes after the addition of the alcohol.

Gooch Crucibles.—The Gooch crucibles are prepared by placing one layer of No. 40 Whatman filter paper in the bottom, on top of this a thin layer of asbestos, then a second layer of filter paper, and finally a second layer of asbestos. The precipitate is so fine, however, that for the first four or five determinations results will be obtained which are from 3 to 10 per cent low. After this the results are generally accurate within 1 or 2 per cent. It is recommended that four crucibles be prepared as outlined above, and before any quantitative determinations are made, between 300 and 400 mg. of sodium pyroantimonate be filtered through each pad, thus clogging the pores of the filter. The sodium pyroantimonate may be prepared by the addition of 10 c.c. of the reagent and 3 c.c. of 95 per cent alcohol to 2 c.c. of a sodium chloride solution containing from 3 to 5 mg. of sodium per cubic centimeter. After standing about five minutes 60 to 100 mg. of precipitate will be obtained. This is transferred to the Gooch crucible and washed with 30 per cent alcohol. The procedure is repeated until 300 to 400 mg. have been transferred. One set of crucibles has been used over 25 times and at the end of that time

only moderate suction was necessary to produce the optimum rate of filtration. Thus, if four crucibles are prepared in the manner described above, over 100 sodium determinations may be made before it is necessary to make a new set.

Filtration.—The precipitate is transferred to the Gooch crucible with the aid of a rubber-tipped rod. After the pad has become soaked, moderate suction is used so that the fluid goes through at the rate of 10 to 15 drops per minute. When all the fluid has passed through the filter, the rubber-tipped rod is washed off and the small amount of precipitate remaining in the platinum dish transferred to the Gooch by means of 8 to 12 c.c. of 30 per cent. alcohol.

Drying.—The drying is carried out at 110° C. for one hour and then the crucible and contents are placed in a desiccator to cool. At the end of thirty minutes the crucible is weighed.

POTASSIUM IN BLOOD

Method of Clausen [1]

Principle.—Blood or plasma is subjected to "wet" washing to remove organic material and the potassium in the clear solution is precipitated as potassium sodium cobaltic nitrite, $K_2NaCo(NO_2)_6.2H_2O$. This precipitate is oxidized in an acid solution with an excess of potassium permanganate and the excess of permanganate is titrated with oxalic acid.

Procedure.—The blood is ashed in a 200×25 mm. Pyrex glass tube with a mixture of nitric and sulfuric acids. For 2 c.c. of plasma, or 1 c.c. of blood, 5 c.c. of a mixture of sulfuric acid, 1 part, nitric acid, 20 parts, are sufficient. The tube arranged with a fume absorber as for a Folin micro-Kjeldahl determination is heated by a micro-burner, adjusted so that boiling is slow, for about one-half hour. Foaming and bumping can be largely prevented by the use of a short piece of platinum wire sealed through the bottom of the tube, serving as a heat-conductor and boiling focus. After one-half hour the excess of HNO_3 is rapidly evaporated. The remaining drop of sulfuric acid darkens considerably. At this stage, the flame is turned off, and the small amount of HNO_3 condensed on the walls of the tube allowed to run back.

[1] Clausen: Jour. Biol. Chem., 1918, 36, 479.

If this is not sufficient to clear up the H_2SO_4, two to three drops of HNO_3 are added and boiling is resumed until the acid is colorless, when the tube is cooled.

The oxidation mixture is washed into a 50 c.c. beaker, made alkaline to phenolsulfonephthalein with 10 per cent NaOH, and evaporated to dryness on the water bath. A few drops of glacial acetic acid are added, until the mixture is acid; then 1 c.c. cobaltic nitrite reagent is added, and evaporation continued until crystals of Na_2SO_4 appear. The mixture is then cooled. Dehydration by means of the Na_2SO_4 results in very complete precipitation of the $K_2NaCo(NO_2)_6$.

The precipitate of potassium sodium cobaltic nitrite is filtered off on a Gooch crucible with a fairly thick asbestos mat. Filter paper must not be used, because during the subsequent boiling with 1 per cent NaOH, considerable reducing substance is formed from it. Just before the mat is sucked dry, a suspension of $BaSO_4$ is poured onto it. This not only fills up the larger pores, but serves as an excellent test of the mat. Before the water has all passed through the mat, the contents of the beaker containing the potassium sodium cobaltic nitrite are poured on. The beaker is rinsed with 4 to 5 c.c. of cold water, and the rinsings are poured onto the nearly dry mat. Such washing, with 2 to 3 c.c. of water, is repeated six times. The mat when almost dry is transferred to the beaker, and all adhering particles are washed from the crucible to the beaker with 9 to 10 c.c. of water. One c.c. of 10 per cent NaOH is added, and the contents of the beaker are heated to boiling, then cooled. The dark brown mixture is made up to exactly 25 c.c. and centrifuged. Twenty c.c. of the clear fluid are pipetted off into a 150 c.c. Erlenmeyer flask, 5 c.c. of 1:4 H_2SO_4 added, and titration with 0.02 N $KMnO_4$ as described above is at once carried out. Blanks, run frequently, are usually small (0.05 to 0.10 c.c.). The potassium value of the permanganate is determined by analysis of the standard solution B. This value should be determined frequently, but does not vary greatly from day to day. Calculations are based on the values thus obtained rather than on a theoretical factor.

Estimation of Potassium in Pure Solutions

The reagents are:

1. *Sodium Cobaltic Nitrate Reagent.*—This is prepared from two stock solutions which keep well several months.

A. Cobalt nitrate	50 gms.
Glacial acetic acid	25 c.c.
Distilled water to	100 c.c.
B. Sodium nitrite	100 gms.
Distilled water to	200 c.c.

Six parts A and 10 parts B are mixed. A rapid current of air is passed through the solution for several hours to remove the fumes of oxides of nitrogen. Then the dark-brown fluid is kept on ice two days. Invariably some yellow precipitate forms, owing to traces of potassium and ammonium in the reagents. The filtered reagent keeps well for several weeks, if in a dark bottle in an ice box.

2. *Potassium Permanganate.*—This is prepared by diluting 0.1 N $KMnO_4$ to about 0.02 N and boiling under a funnel reflux condenser for two to three hours. After one to two days, the solution is decanted from the manganese oxides which have separated, and preserved in a cool dark place. Such solutions keep well several weeks.

3. *Standard Potassium Solutions.*

A. Standard, 1 c.c. = 10 mg. potassium.	
Pure KCl	9.546 gms.
HCl (concentrated)	1 c.c.
Water to	500 c.c.
B. Standard, 2 c.c. = 1 mg.	
Standard A	25 c.c.
HCl (concentrated)	1 c.c.
Water to	500 c.c.

The potassium chloride is recrystallized several times, then fused. The hydrochloric acid is added to prevent growth of moulds.

In conclusion, it is to be emphasized that the reagents must be tested for the presence of potassium. One sample of c.p. NaOH purified by alcohol contained nearly 0.05 per cent potassium.

The sodium citrate used as an anticoagulant must also be tested for the presence of potassium.

POTASSIUM IN BLOOD

Method of Kramer [1]

Principle.—Similar to that in the method of Clausen, page 191.

Procedure.—Blood is collected by puncture of the median basilic, median cephalic, or external jugular vein. Whole blood is collected directly into a weighed platinum crucible and rapidly weighed. Plasma or serum may be collected under oil as in the Van Slyke and Cullen determination of the CO_2 combining power of blood plasma. To obtain plasma, potassium-free ammonium oxalate or oxalic acid may be used as anticoagulant. Although ammonium salts react with sodium cobalti-nitrite, the ammonia is completely volatilized during the ashing. To obtain serum, the blood is collected under oil in a centrifuge tube and allowed to remain in the ice box until the serum has separated. If the blood is collected in this manner or in a clean, dry test-tube the danger of hemolysis will be reduced to a minimum.[2]

Potassium Method

One c.c. of blood, 3 to 5 c.c. of clear plasma, or an equal amount of serum is dried in a platinum dish over the steam bath, then in the incubator at 110° C. for about one-half hour. The dish or crucible is then placed in a flat-bottomed quartz dish, 10 cm. in diameter and 6 cm. deep, in the bottom of which are placed several pieces of porcelain. The outer dish is then heated with the low flame of a large Méker burner until fumes begin to come off. The heating is continued until no more fumes are given off, when the flame is turned on full until the charred material is immobile. The large dish is then covered with a quartz plate and heating continued until the material is completely ashed.[3] The platinum

[1] Kramer: Jour. Biol. Chem., 1920, 41, 263.

[2] Owing to the large amount of potassium present in corpuscles even a moderate amount of hemolysis introduces considerable error.

[3] The ashing often proceeds rapidly at first, then some residual carbon is left which does not readily become oxidized. At this stage the ash is dissolved in a little concentrated hydrochloric acid, evaporated over the steam

dish is then removed, allowed to cool, and the ash dissolved in 0.5 c.c. of water with the aid of one or two drops of glacial acetic acid. 0.5 to 1 c.c. of sodium cobalti-nitrite reagent is then added, drop by drop, with stirring and the mixture allowed to stand for at least ten minutes. During this time a Gooch crucible is prepared. One or two pieces of hardened filter paper are placed at the bottom then asbestos emulsion is poured in, and the pad sucked dry. When finished the pad should be at least 2 mm. in thickness and should be washed with a large quantity of water. The precipitate is transferred to the wet pad. The suction should be regulated so that the water runs through drop by drop; the rest of the precipitate is washed onto the pad with small portions of cold water. It is then washed repeatedly with small portions of water until the washings return perfectly clear. Precipitate and pad are then transferred *en masse* to a 50 c.c. beaker, and the crucible is washed with a little water.[1] The paper is then removed with a forceps and washed clean with distilled water. Not more than 10 c.c. of water need be used for complete transference of asbestos pad and precipitate. Twenty-five c.c. (an excess) of 0.01 N potassium permanganate and 5 c.c. of 25 per cent sulfuric acid are added. The mixture is stirred, heated over the steam bath for just three minutes, and sufficient 0.01 N oxalic acid is then added to decolorize the solution completely. The material is at once titrated back to a permanent pink with 0.01 N $KMnO_4$ solution. The total number of c.c. of 0.01 N $KMnO_4$ − number of c.c. of 0.01 N oxalic acid × 0.071 = mg. of potassium in the sample.

A blank may be done but the correction is insignificant as a rule.

Preparation of the Reagents.—*25 per cent sulfuric acid* is made by diluting chemically pure concentrated sulfuric acid with water. It should be tested with permanganate for the presence of organic matter.

Solution A.—Fifty gms. of cobalt nitrate crystals (J. T. Baker) are dissolved in 100 c.c. of water and to this solution 25 c.c. of glacial acetic acid are added.

bath, then dried in the incubator at 105° C., and heated until a white crystalline ash is obtained.

[1] The pad need not be sucked dry. By means of a glass rod it may be dislodged by a half turn and can then be transferred as a whole including the entire precipitate.

Solution B.—Fifty gms. of c.p. sodium nitrite (potassium-free) (J. T. Baker) are dissolved in 100 c.c. of water. Mix six volumes of Solution A and ten volumes of Solution B. An evolution of nitric oxide gas occurs at once. Air is drawn through the solution until all the gas has passed off. The reagent is then allowed to stand in the ice box for at least twenty-four hours. It is best filtered before using.

The cobalt nitrate and sodium nitrite need be weighed only roughly. If kept in the ice box the reagent will keep for at least a month and often much longer. Although 1.5 c.c. of reagent will precipitate as much as 20 mg. of potassium it is best to use about 0.5 c.c. where 1 mg. of potassium or less is thought to be present. For quantities between 1 to 3 mg. 1 c.c. of reagent will suffice.

0.01 N Potassium Permanganate Solution.—0.01 N potassium permanganate solution is prepared from a N or 0.1 N solution by appropriate dilution. The solution is standardized against a known 0.01 N oxalic acid solution. The latter is made from a N oxalic acid solution.

Na, Ca, Mg, Fe, sulfates, chlorides, and nitrate do not interfere. Ammonia must be removed. Although such small amounts of phosphates as occur in normal blood do not interfere with the accuracy of the potassium determination, nevertheless, as has recently been shown, the inorganic phosphates may be markedly increased in the blood serum in certain pathological conditions. Hence it may be necessary to remove them. The following method has been found satisfactory. The solution of blood or serum ash acidified with hydrochloric acid is heated on the steam bath for a few minutes. Two c.c. of 2 per cent $BaCl_2$ solution are added drop by drop followed after a few minutes by 0.5 c.c. of concentrated ammonia. Fifteen c.c. of a saturated solution of ammonium carbonate in a mixture of equal volumes of concentrated ammonia and 95 per cent alcohol are added drop by drop with stirring. After one-half hour the precipitate, which contains practically all the barium, calcium, magnesium, sulfate, and phosphate, is filtered through ash-free filter paper and washed several times with the precipitating reagent.

When the filtrate which contains the sodium and potassium has been evaporated to a small volume, a few drops of concentrated hydrochloric acid are added. Evaporation to dryness is continued

and completed in the incubator at 110° C. The residue is then heated to constant weight by the method of Stolte and the sodium and potassium are weighed as the chlorides. When combined with the cobalti-nitrite method for potassium, this procedure may be used as a rapid method for the indirect determination of sodium. It is also possible to weigh the combined chlorides of sodium and potassium, determine the total chlorides, and calculate the amount of potassium and sodium.

IRON IN BLOOD

Method of Berman [1]

Principle.—The iron held in combination in blood is split off by the action of concentrated hydrobromic acid. The iron is oxidized to the ferric condition and the organic matter is destroyed by potassium permanganate. The resultant solution is mixed with a solution of ammonium sulphocyanate in water and acetone and the color is compared with that of a standard iron solution similarly treated.

Procedure.—Blood is obtained in the usual manner for determining hemoglobin with the Sahli apparatus, by pricking the finger or ear lobe. To 0.040 c.c. of blood, measured in a calibrated pipette, mixed with 2 c.c. of water and 0.2 c.c. of 0.1 N HCl, 2 c.c. of 0.1 N $KMnO_4$ are added. Then the mixture is placed in a water bath for about two minutes, when a brownish red coagulum results, supernatant above a colorless or slightly yellowish fluid. To this two drops of concentrated hydrobromic acid are added. The tube is returned to the water bath for another two minutes. The product should be a water-clear fluid containing a few whitish flakes. The solution is filtered through acid-washed filter paper into a long narrow graduate. Washings are added until the filtrate has reached the 5 c.c. mark. Then 5 N NH_4CNS is added up to 10 c.c., and acetone up to 20. The graduate is stoppered, and the fluids are mixed. A shrinkage of volume occurs. Acetone is added again to the 20 c.c. mark, and the graduate is stoppered. A salmon-red color is produced.

The standard, which may be prepared at the same time as the blood, is made by adding two drops of concentrated hydrobromic

[1] Berman: Jour. Biol. Chem., 1918, 35, 231.

acid to 2 c.c. of the standard iron solution containing 0.009 mg. of Fe per c.c., and keeping it in the water bath for two to four minutes. It also is made up to 5 c.c. with water, to 10 c.c. with 5 N NH_4CNS, and to 20 c.c. with acetone in a narrow graduate, which is stoppered and shaken. After the shrinkage incidental to the mixture of fluids, acetone is added up to 20 c.c. and the graduate is stoppered and shaken.

Allow both graduates to stand for five minutes.

Comparison may be best made in a Duboscq or Kober colorimeter, with the standard set at 10. The calculation is $\frac{10}{R} \times 45 =$ number of mg. of Fe per 100 c.c. of blood. The comparison must be made as quickly as possible, as the acetone evaporates on exposure to the air, although about equally in both cups.

Obtaining Blood.—The ubiquity of iron makes special care to avoid outside contamination necessary in obtaining the blood. The finger or ear lobe should be well cleansed with water and ether before pricking. A free flow, without squeezing, should be obtained. Citrate and oxalate do not interfere with the determination, so that blood drawn by venepuncture and so treated can be analyzed for iron by the method. Corpuscles and plasma should, of course, be well mixed before being drawn into the pipette. If possible, the blood should be drawn in the morning before breakfast in order to have as constant a condition of the blood volume as possible.

The pipette should be calibrated by weighing the water it holds. The blood should always be rinsed into the 2 c.c. of water plus 0.2 c.c. of 0.1 N HCl. The least amount of blood with which the determination may be made satisfactorily is 0.040 c.c.

Hydrobromic Acid.—The hydrobromic acid is the pure concentrated hydrobromic acid, 34 per cent strength. The drops delivered from the pipette are of approximately 0.1 c.c. each. If the two drops added to the permanganate coagulum do not clear it up on boiling, another drop may be added to it as well as to the standard.

Permanganate.—The permanganate used should be iron-free, as determined by a blank. If it is not, repeated recrystallization of the commercial product is necessary. Reliable commercial firms, however, furnish an iron-free permanganate. The 0.1 N solution should be kept from the light in a dark bottle.

Standard Iron Solutians.—These are most easily prepared from

chemically pure ferric chloride ($FeCl_3.6H_2O$). The standard as prepared and used contains an amount of iron equal to that in the hemoglobin of 0.040 c.c. of blood when the hemoglobin content is 100 per cent in the Haldane scale; i.e., 13.6 gms. of hemoglobin per 100 c.c. of blood binding 18.5 c.c. of oxygen. The figure 13.6 gms. of hemoglobin per 100 c.c. as a basis may be derived from Hüfner's [1] finding that 1 gm. of hemoglobin will combine with 1.36 c.c. of oxygen. This has been confirmed by a number of investigators. If 1 gm. of hemoglobin will combine with 1.36 c.c. of oxygen, 18.5 c.c. of oxygen will combine with 13.6 gm. of hemoglobin. Butterfield,[2] as others before him, has shown that the iron content of 1 gm. of hemglobin is constant, lying between 0.33 and 0.34 per cent, that is, one three-hundredth of the molecule. One three-hundredth of 13.6 gives 45 mg. of Fe per 100 c.c. of standard blood. This is the standard used in the determination of iron. It may be made up from a concentrated solution of ferric chloride, 4.355 gms. of $FeCl_3.6H_2O$ in a liter of water, by taking 1 c.c. and making up to 100 c.c. with water, which is the standard solution containing 0.009 mg. of Fe per c.c.

If the hemoglobin is much higher than the normal, other standards can be used, corresponding respectively to 25, 35, 55, and 65 mg. of Fe per 100 c.c. of blood. In the note on the colorimetric comparison reference will be made to this.

Ammonium Sulfocyanate.—This is prepared as 5 N in order to have a concentrated solution which reduces the degree of dissociation of the ferric sulfocyanate into the colorless ions. It should be kept in a dark bottle away from the light.

Comparison in Colorimeters.—Duboscq and Kober colorimeters proved suitable for this work. A comparison of the standard itself should always be carried out quickly. Readings can be made in daylight, preferably from a north window, or with artificial light filtered through "daylight glass." The average of at least four readings, quickly done before the acetone evaporates, is taken. As in all colorimetric readings, if the reading falls below 12 or above 8 (the standard being at 10) a reading should be made with one of the other standards mentioned above. Indeed, the standard to be used should depend upon the hemoglobin reading; e.g., with 50 per cent hemoglobin the standard corresponding to 25 mg. of

[1] Hüfner, G., Arch. Physiol., 1894, 130.

[2] Butterfield, E. E., Z. physiol. Chem., 1909, 62, 173.

Fe per 100 c.c. of blood should be used (that is 0.005 mg. of Fe per c.c. of standard iron solution). For every 5 mg. above or below the standard of 45 mg. of Fe per 100 c.c., 0.001 mg. is added to or subtracted from the standard of 0.009 mg. of Fe per c.c. Ordinarily, the latter standard, corresponding to 45 mg. of Fe per 100 c.c., is used.

CHLORIDES IN BLOOD

Method of Whitehorn,[1] *Supplementary to Method of Folin and Wu for Blood Analysis* (*see this Manual, page* 83)

Principle.—Proteins in blood are precipitated by the use of tungstic acid. The chlorides in the filtrate thus prepared are precipitated as silver chloride from a known quantity of silver nitrate and the excess of silver nitrate is determined by titration with sulphocyanate, employing ferric alum as an indicator.

I. **Preparation of Protein-Free Filtrates.**—The filtrate is prepared by the use of the same reagents as have been described in detail by Folin and Wu (see this Manual, page 83) for the determination of non-protein nitrogen, urea, uric acid, creatinine, creatine, and sugar. Because even slight variations in the chlorides are significant, great accuracy is necessary. The author customarily uses volumetric flasks in order to insure an accurate 1:10 dilution. The method is applicable, without alteration, to either whole blood or plasma. An amount of filtrate equivalent to 1 c.c. of blood or plasma is needed. Less may be used but with proportionate loss of accuracy.

II. **Determination of Chloride Content of Filtrate.** (*a*) **Reagents Required.**—1. *Silver Nitrate Solution.* (*M/35.46*).

2. *Potassium* (*or ammonium*) *sulfocyanate* (*M/35.46*).

3. *Powdered ferric ammonium sulfate* ($FeNH_4(SO_4)_2$).

4. *Concentrated nitric acid* (HNO_3 *of specific gravity 1.42*).

(*b*) **Procedure.**—Pipette 10 c.c. of the protein-free filtrate into a porcelain dish. Add with a pipette 5 c.c. of the standard silver nitrate solution and stir thoroughly. Add about 5 c.c. of concentrated nitric acid, mix, and let stand for five minutes, to permit the flocking out of the silver chloride. Then add with a spatula an abundant amount of ferric ammonium sulfate (about 0.3 gm.)

[1] Whitehorn: Jour. Biol. Chem., 1921, 45, 449.

and titrate the excess of silver nitrate with the standard sulfocyanate solution until the definite salmon-red (*not yellow*) color of the ferric sulfocyanate persists in spite of stirring for at least fifteen seconds.

(*c*) **Calculation.**—5.00 − titer (in c.c.) = mg. of Cl per c.c. of blood (or plasma).

Since each cubic centimeter of thiocyanate solution used is equivalent to 1 c.c. of silver nitrate solution, the difference between the volume of silver nitrate solution taken and the excess determined by the titration, that is 5 − titer, represents the volume which reacted with chloride at the ratio of 1 c.c. to 1 mg. of Cl. And the 10 c.c. of filtrate taken represents 1 c.c. of blood (or plasma).

To convert Cl figures into NaCl figures divide by 0.606. The same result may be more easily obtained by the following rule: To obtain mg. NaCl per 100 c.c., divide mg. Cl per liter by 6, and subtract 0.001 of the result. Conversely, to obtain mg. Cl per liter, add to mg. NaCl per 100 c.c. 0.001 of itself and multiply by 6.

Preparation of Reagents. *Silver Nitrate Solution.*—Dissolve 4.791 gms. of c.p. silver nitrate in distilled water. Transfer this solution to a liter volumetric flask and make up to the mark with distilled water. Mix thoroughly and preserve in a brown bottle. One c.c. = 1 mg. Cl. (It is to be noted that the silver nitrate and nitric acid are not added to the protein-free filtrate simultaneously. To do so may result in the mechanical enclosure of silver nitrate solution within the curds, and a consequent error in the positive direction.)

Because sulfocyanates are hygroscopic, the standard solution should be prepared volumetrically. As an approximation about 3 gms. of KCNS or 2.5 gms. of NH_4CNS should be dissolved in a liter of water. By titration under the conditions specified under "Procedure" and by proper dilution prepare a standard such that 5 c.c. are equivalent to 5 c.c. of the silver nitrate solution.

The solid ferric alum is used rather than a solution, in order to insure a very high concentration in the mixture to be titrated. It is powdered in order to facilitate its solution.

PLASMA CHLORIDES

Method of Van Slyke and Donleavy[1]

Principle.—Precipitation of the proteins and titration of the chlorides with a standard potassium iodide solution.

Procedure.—Two c.c. of oxalate or citrate plasma are drawn into a dry pipette calibrated to contain 2±0.005 c.c. (the pipette must weigh 1.994±0.005 gm. more when filled with water at 20° than when empty and dry). The plasma is run into a 50 c.c. measuring flask half full of water, and the pipette is rinsed by drawing the water up into it twice. Ten c.c. of the standard silver nitrate-picric acid solution are added, and the mixture is diluted to the 50 c.c. mark and shaken at intervals for several minutes until coagulation is complete. The addition of a drop or two of caprylic alcohol prevents foaming and facilitates coagulation. The solution is passed through a dry, chloride-free filter, the first portion of filtrate being passed through, if necessary, a second time to remove turbidity completely. Twenty c.c. duplicate portions of the filtrate are measured with a calibrated pipette into 100 c.c. Erlenmeyer flasks, 4 c.c. of the starch-citrate indicator solution are added to each, and the standard KI is run in from a burette until a permanant blue end-point is reached.

If it is desirable to use less than 2 c.c. of plasma, 1 c.c. measured within ±0.002 c.c. may be precipitated with 5 c.c. of the standard silver solution and diluted to 25 instead of 50 c.c., the filtrate yielding only one 20 c.c. portion instead of duplicates.

The End-point.—Only a permanent and unmistakable blue is taken as the end-point. If the iodide is run in rapidly near the end of the titration, iodine may be formed more rapidly than silver nitrate precipitates it, and a false end-point reached which disappears after a few seconds shaking. If, toward the end of the titration, the iodide is added slowly the blue shade caused by each drop disappears as soon as the solution is rotated, until the genuine end-point is reached. The latter is permanent, and in fact deepens with time.

Standard Solutions. 1. *Silver Nitrate Solution.*—An acid M/29.25 solution of silver nitrate, 1 c.c. of which is equivalent to 2 mg. of NaCl.

[1] Van Slyke and Donleavy: Jour. Biol. Chem., 1919, 37, 551.

$AgNO_3$	5.812	gms.
HNO_3 (sp. gr. 1.42)	250	c.c.
Picric acid	7.5	gms.
Water to	1000	c.c.

II. *Starch Solution.*—A solution, for use in the final titration, containing sodium citrate, sodium nitrite, and starch, which substances respectively regulate the acidity, provide an oxidizing agent for the iodide, and serve as indicator.

Sodium citrate ($Na_3C_6H_6O_7+5\frac{1}{2}H_2O$)	446	gms.
Sodium nitrite	20	gms.
Soluble starch	2.5	gms.
Water to	1000	c.c.

The starch is first dissolved in about 500 c.c. of boiling water, to which the citrate and nitrite are then added, the solution being finally made up to 1 liter. It should be emphasized that when the starch is dissolved the solution must be not merely heated but boiled for several minutes, or it will not give a satisfactory end-point. If starch other than the soluble variety is used the boiling should continue for one hour. Four c.c. of this solution contains sufficient citrate to react with the acid in 1 c.c. of nitric acid of 1.42 specific gravity, the resulting solution having the optimum acidity for the production of the blue starch-iodine end-point.

III. *Potassium Iodide Solution.* — A solution of M/73.1 potassium iodide of which 1 c.c. is equivalent to 0.8 mgm. of NaCl.

KI	2.27	gms.
Water to	1000	c.c.

To standardize this solution proceed as follows: The KI solution is made up to contain 2.4 gms. KI per liter and diluted to the extent indicated by a preliminary titration. Five c.c. of the silver nitrate solution, measured with a pipette which has an error not greater than 0.01 c.c. (delivers 4.97 gms. of water at 20°), are mixed with 5 c.c. of the starch solution and 5 c.c. of water, and iodide is run in from a burette to a permanent blue end-point. The amount required should be 12.65 c.c., 12.50 c.c. being required to precipitate the standard silver solution, and 0.15 c.c. additional to give the end-point. The fact that 0.15 c.c. of excess KI solution is required to give the end-point does not detract from the accuracy of the titration, because, with a given volume of the solution, the required excess is constant and sharply defined.

Calculation.—The calculation is very simple when standard solutions of the above concentration are used. The 20 c.c. of filtrate employed for titration represent 0.8 c.c. of plasma, and the unprecipitated portion of the amount of silver nitrate equivalent to 8 mgm. of NaCl or 10 mgm. per c.c. of plasma. Each c.c. of KI used in the titration is equivalent to 1 mgm. of NaCl per c.c. of plasma. Hence the calculation simplifies to—

$$\left.\begin{array}{l}\text{Mg. NaCl per c.c. plasma or}\\ \text{Gm. NaCl per liter } \text{“}\end{array}\right\} = 10.15 - \text{c.c. KI.}$$

The use of 10.15 instead of 10 c.c. is due to the fact that 0.15 c.c. excess of KI solution is required to give the end-point. If the 0.15 c.c. of excess iodide required to give the end-point were neglected in the calculation the error would be partially eliminated by neglecting it also in the standardization. It is as simple, however, to allow for it in the calculation and thereby eliminate it entirely.

Remarks.—In the determination of plasma chlorides it is essential that all pipettes, burettes and measuring flasks be accurately calibrated since this apparatus as usually obtained from dealers is not sufficiently accurate to be employed for this determination without being checked.

CHLORIDES IN WHOLE BLOOD OR PLASMA

Method of Meyers and Short [1]

Principle.—Proteins are precipitated from the blood and the chlorides in the filtrate are titrated according to the Volhard method.

Procedure.—Three c.c. of whole blood (or plasma) are added to 27 c.c. of distilled water in a 50 c.c. centrifuge tube. About 0.5 gm. of dry picric acid is then added and the mixture stirred until protein precipitation is complete and the whole mixture turns a bright yellow color. The precipitate is now thrown down by centrifuging for a few minutes at moderate speed and the clear supernatant fluid decanted into a clean dry beaker. (If any particles remain suspended the fluid should be filtered.)

Twenty c.c. are then pipetted into a clean dry 50 c.c. centrifuge tube and 20 c.c. of standard $AgNO_3$ solution, of such strength

[1] Meyers and Short: Jour. Biol. Chem., 1920, 44, 47.

that 1 c.c. is equivalent to 1 mg. of NaCl (Solution 1), and 10 c.c. of the dilute acidified ferric alum indicator added (Solution 2). The contents are stirred to insure thorough mixing and the AgCl precipitate is thrown down in the centrifuge. The clear supernatant fluid is decanted into a clean, dry beaker and 20 c.c. portions are pipetted into each of two small porcelain evaporating dishes for duplicate titrations.

The titration is made with ammonium thiocyanate solution of such strength that 2 c.c. are equivalent to 1 c.c. of the $AgNO_3$ solution (Solution 3). The end-point is definite and consists of the first permanent tinge of reddish brown which extends throughout the mixture. Some experience may be necessary before the end-point is always recognized, but thereafter there need be no difficulty in obtaining exact duplicate titrations. Passing the end-point by one drop will introduce an error ordinarily of about 0.5 per cent in estimating chlorides in 100 c.c. of blood or plasma.

Calculation.—The calculation is $10-\left(\frac{\text{Titer}}{2}\times\frac{5}{4}\right)\times 100=\text{mg.}$ of NaCl in 100 c.c. of whole blood or plasma.

Solutions Required.—1. *Silver nitrate* of such strength that 1 c.c. is equivalent to 1 mg. of sodium chloride, 2.904 gms. to 1000 c.c. It may be prepared by a 1 to 10 dilution of the silver nitrate employed in the Volhard-Harvey method for urine.

2. *Acid ferric alum indicator* prepared by dissolving 100 gms. of crystalline ferric ammonium sulfate in 100 c.c. of 25 per cent nitric acid and adding four parts of distilled water. This is one-fifth the strength employed for urine.

3. *Ammonium thiocyanate* of such strength that 2 c.c. are the equivalent of 1 c.c. of the above silver solution, or one-twentieth of the strength employed for urine. It contains approximately 0.65 gm. of the thiocyanate to 1000 c.c.

To simplify the test further it is possible to combine Solutions 1 and 2, two parts of No. 1 and one part of No. 2 being used.

CHLORIDES IN WHOLE BLOOD

Method of Austin and Van Slyke [1]

Principle.—Removal of proteins and titration of the chlorides.

Procedure.—Take 3 c.c. of blood with 15 c.c. of water in a 60 c.c. flask. Add 30 c.c. of saturated picric acid solution and

[1] Austin and Van Slyke: Jour. Biol. Chem., 1920, 41, 345.

sufficient water to bring the volume to 60 c.c. (27 c.c. of water in all, and 30 c.c. of picric acid may be measured from burettes if a 60 c.c. measuring flask is not available). The contents of the flask are mixed, and after ten minutes are filtered. To 40 c.c. of the filtrate 10 c.c. of the standard silver nitrate solution are added with 2 drops of caprylic alcohol. The solutions are thoroughly mixed, and preferably allowed to stand over night to allow the AgCl to coagulate and settle. The supernatant solution is decanted through a small filter paper and 20 c.c. are titrated in accordance with the method of Van Slyke and Donleavy, see page 202. The calculation is also the same since the 20 c.c. of filtrate titrated in this case, as in the method of Van Slyke and Donleavy, represent 0.8 c.c. of the original material, blood or blood plasma.

Solutions.—I. *Silver Nitrate Solution.*—An acid M/29.25 solution of silver nitrate, 1 c.c. of which is equivalent to 2 mg. of NaCl.

$AgNO_3$	5.812	gms.
HNO_3 (sp. gr. 1.42)	250	c.c.
Water to	1000	c.c.

II. *Starch Solution.*—Same as for method of Van Slyke and Donleavy, see page 202.

III. *Potassium Iodide Solution.*—Same as that of Van Slyke and Donleavy, see page 202.

HEMOGLOBIN DETERMINATION

Haldane's Method [1]

Principle.—The hemoglobin of the blood is changed to CO-hemoglobin and this diluted blood specimen is compared with a standard solution of CO-hemoglobin.

Procedure.—With a 20 c.mm. pipette measure the blood into the graduated tube containing a small volume of water. After the blood has been added, but before mixing, a piece of glass tubing connected with a gas jet is introduced into the free part of the tube, so that air is instantly displaced from the tube. The gas tube is then withdrawn while the gas is still flowing and the top of the graduated tube at the same time quickly closed with the finger. The liquid is then made to run up and down the tube

[1] Haldane: Jour. Physiol., 1900–01, 26, 497.

about a dozen times, so that the hemoglobin becomes thoroughly saturated with CO and the full pink tint of CO-hemoglobin appears. Water is then added drop by drop from the dropping pipette supplied, until the point is reached when the tints appear to be equal. The percentage is read off on the tube after one-half minute has been allowed for the liquid to run down. Another drop is now added, and if necessary, another, until the tints again appear unequal. The mean of the readings which gave equality is taken as the correct result.

In comparing the tints of the two tubes, it is best to hold them up against the light from the sky, or toward an opal glass globe when artificial light is used. The precaution must always be taken of repeatedly transposing the tubes from side to side during the observations; otherwise very considerable errors may arise.

The error of the method is not greater than 1 per cent.

Preparation of Standard CO-Hemoglobin.—The standard solution for the hemoglobinometer is a 1 per cent solution, saturated with coal gas, of ox or sheep blood of the average oxygen capacity of normal adult males (18.5 per cent). In making the standard solution the tube should be sealed and no air should be in the tube, otherwise the standard will gradually change its tint.

HEMOGLOBIN DETERMINATION

Palmer's Method [1]

Principle.—Hemoglobin of the blood is transformed into CO-hemoglobin and comparison is then made in the colorimeter with a standard solution of CO-hemoglobin.

Procedure.—Fill a 10 c.c. volumetric flask about half full of the dilute ammonia (see below). Draw blood to the mark in the 0.1 c.c. pipette. Transfer to the volumetric flask containing the ammonia water, drawing the solution into the pipette two or three times to wash out all blood. Next fill the volumetric flask to the mark with the ammonia solution. Transfer contents to a large-sized test-tube and bubble briskly illuminating gas or carbon monoxide through the hemoglobin solution for at least thirty seconds (this operation should, of course, be carried out in a hood

[1] Palmer: Jour. Biol. Chem., 1918, 33, 119.

or otherwise so conducted that danger of carbon monoxide poisoning is avoided). Compare in colorimeter against standard 1 per cent carbon monoxide hemoglobin solution.

Apparatus and Reagents.—*0.1 c.c. pipette* (*calibrated to contain 0.1 c.c.*).

10 c.c. volumetric flask.

Dilute ammonia solution (4 c.c. strong ammonia in 1 liter of water).

Colorimeter.

Standard hemoglobin solution (same as in Haldane's Method).

Suggestions.—1. A standard made up to contain a 20 per cent solution of blood, having an oxygen capacity of 18.5 per cent, is kept in the ice-chest, saturated with CO (illuminating gas). From this a 1 per cent solution for routine use may be made. Seal in the cork with paraffin. This concentrated solution will keep for months.

2. A standard 1 per cent solution for routine use is made up in 100 and 200 c.c. lots and kept sealed in a bottle which should be protected from light. With ordinary care, if kept in the ice-chest and protected from light, it will keep for weeks. At room temperature it keeps for about a week.

3. In making standards or dilutions always use the specified dilute ammonia solution.

HEMOGLOBIN DETERMINATION

Method of Cohen and Smith [1]

Principle.—The hemoglobin of blood is transformed into acid hematin and this solution compared in a colorimeter with a standard solution of acid hematin.

Procedure.—From a freely flowing source of blood, 0.02 c.c. is measured by means of a calibrated Sahli pipette into 6 c.c. of 0.1 N hydrochloric acid. The blood pipette is rinsed out by drawing the acid solution into it several times. Blood very low in hemoglobin may require a double sample, i.e., 0.04 c.c. of blood in 6 c.c.

[1] Cohen and Smith: Jour. Biol. Chem., 1919, 39, 489.

of acid, in order to give a dilution having the most satisfactory color for comparison with the standard. After the sample is added to the acid, the mixture must be allowed to stand, preferably in a warm place, for at least ten minutes for the full color to develop. Readings taken sooner will be too low.

In cold weather, hemolysis and color development take place more slowly, and the application of gentle heat will hasten the process. As a routine procedure, immersion of the tube in a warm water bath is recommended. The color comparison may be made in either the Autenrieth-Hellige or the Duboscq colorimeter with a standard acid hematin solution. The average of at least several readings is taken. The calculation is simple and described below for each instrument.

Standard and Calculation for the Duboscq Colorimeter.—The standard for comparison is a 0.5 *per cent* blood solution, which is set at 10 upon the Duboscq scale. Hence the per cent hemoglobin = $\frac{1.5\times10\times100}{\text{Reading}}$. It is desirable to make an actual calibration of the instrument for the solutions to be examined.

Standard and Calculation for the Autenrieth-Hellige Colorimeter.—The standard for comparison is a 1.0 *per cent* blood solution. Experience has pointed to the necessity for different concentrations of standard solutions in each kind of instrument in order to secure most satisfactory color comparisons. It may be found that the scale on the Autenrieth colorimeter may be inaccurately placed. There is another source of error that cannot be corrected by a mere resetting of the scale. The glass wedge containing the standard is not mathematically perfect; therefore for accurate work, this wedge containing the 1 per cent standard should be calibrated against solutions of known amounts of blood in 0.1 N HCl contained in the small cup. Thus a curve may be constructed from which may be read at once the percentage hemoglobin corresponding to a given reading in the scale. This calibration takes a short time, and holds good for that wedge and instrument as long as other conditions are maintained.

For the conditions given here, if the instrument were perfectly constructed, the calculation would be $\frac{3.0\times10\times100}{\text{Reading}}$ = per cent hemoglobin, but this relation holds for only a portion of the scale (between 3.0 and 8.5).

Preparation of the Acid Hematin Standard

A quantity of blood (usually 50 c.c.) is obtained, carefully defibrinated, and then strained through gauze. The oxygen capacity of this blood is then determined by the Van Slyke method. Accepting the Haldane figure of 18.5 volume per cent for the oxygen capacity of normal blood (corresponding to approximately 14 gms. of hemoglobin per 100 c.c.) the blood is diluted with 0.1 N HCl so as to make a 20 per cent solution of a blood with an oxygen capacity of 18.5 volume per cent. That is, if the blood has an oxygen capacity of 18.5 volume per cent, 20 c.c. of it are diluted to 100 c.c. with 0.1 N HCl; if the oxygen capacity is 22 volumes per cent then 16.7 c.c. $\frac{18.5 \times 20}{22}$ of it are diluted 100 c.c. with the acid. The 20 per cent solution of blood thus obtained is well mixed and stored in a glass-stoppered bottle, preferably in a cool spot away from the light. This constitutes the stock solution from which the comparison standard is made. Such a stock solution will not deteriorate for at least three months, provided contamination by molds is avoided. Sahli suggests saturating the acid with chloroform to keep molds from developing in the solution. No other unusual precaution for the preservation of this solution seems to be needed; but before using, it should be thoroughly shaken.

The comparison standard for use in the colorimeter is made by diluting 5 c.c. of the stock solution to 100 c.c. with 0.1 N HCl to make a 1 per cent standard; or 2.5 c.c. of the stock solution to 100 c.c. for a 0.5 per cent standard. Where routine determinations are to be made, it is desirable to have the standards made fresh at least once a week.

Where determinations of the oxygen capacity of the blood are not readily available, one can make the stock solution from crystallized hemoglobin.

METHEMOGLOBIN IN BLOOD

Method of Stadie [1]

Principle.—Both hemoglobin and methemoglobin are changed to cyan-hemoglobin by potassium cyanide. The total amount

[1] Stadie: Jour. Biol. Chem., 1920, 41, 237.

Factors for Calculating Results from Analysis of 2 c.c. of Blood Saturated with Air

Temperature	Air physically dissolved by 2 c.c. of blood. Subtract from gas volume to obtain corrected gas volume representing 0_2 set free from hemoglobin.	Factor by which corrected gas volume is multiplied in order to give hemoglobin in 100 c.c. of blood.
° C.	c.c.	gm.
15	0.037	$34.7\times\frac{B}{760}$
16	0.036	$34.6\times\frac{B}{760}$
17	0.036	$34.3\times\frac{B}{760}$
18	0.035	$34.2\times\frac{B}{760}$
19	0.035	$34.0\times\frac{B}{760}$
20	0.034	$33.9\times\frac{B}{760}$
21	0.033	$33.7\times\frac{B}{760}$
22	0.033	$33.5\times\frac{B}{760}$
23	0.032	$33.4\times\frac{B}{760}$
24	0.032	$33.1\times\frac{B}{760}$
25	0.031	$33.0\times\frac{B}{760}$
26	0.030	$32.9\times\frac{B}{760}$
27	0.030	$32.6\times\frac{B}{760}$
28	0.029	$32.5\times\frac{B}{760}$
29	0.029	$32.3\times\frac{B}{760}$
30	0.028	$32.1\times\frac{B}{760}$

of hemoglobin plus methemoglobin is determined colorimetrically by means of a standard solution of cyanhemoglobin. The hemoglobin in the blood containing the two pigments is next determined gasometrically by Van Slyke's method (this Manual, page 124). This value is subtracted from that obtained for hemoglobin plus methemoglobin determined together as cyanhemoglobin; the difference is the methemoglobin.

Procedure.—Oxalated whole blood is used. Two c.c. of the blood are placed in a 100 c.c. flask and 50 c.c. of water are added which effect hemolysis in a few seconds. 0.5 c.c. of a 0.1 M (3 per cent) solution of potassium ferricyanide is added, and the flask allowed to stand for fifteen to twenty minutes. (It was found that these conditions are optimum for the complete conversion of the hemoglobin to methemoglobin, only the faintest visible hemoglobin band being present at the end of twenty minutes with this amount of potassium ferricyanide.) Five c.c. of a 0.1 per cent potassium cyanide solution are now added. The change to cyanhemoglobin is immediate. Water is added to the mark and the solution compared with a standard of known strength in a colorimeter. The result is the hemoglobin plus methemoglobin, which is expressed as gm. of " total hemoglobin " per 100 c.c. of blood.

A small portion (4 to 5 c.c.) of the blood or hemoglobin solution is aerated in a funnel and its total oxygen capacity determined by the Van Slyke method. Barcroft [1] has shown that under these conditions (180 mm. of oxygen tension, 0 mm. of carbon dioxide tension, and room temperature) the hemoglobin is practically 100 per cent saturated. Therefore the oxygen capacity corresponds to the amount of hemoglobin present, and by dividing by 1.34 (the volume of oxygen combined with 1 gm. of hemoglobin) one obtains the gms. of hemoglobin per 100 c.c. of blood. For the convenience of calculation the factors for the conversion of c.c. of gas combined with 2 c.c. of blood into gms. of hemoglobin per 100 c.c. of blood are given in the table (modified from Van Slyke).

Preparation of Standard.—The standard is prepared from fresh whole oxalated or defibrinated blood which is known to contain no methemoglobin. The hemoglobin content (gms. per 100 c.c.) is determined gasometrically. Five hundred c.c. of standard are made by placing 10 c.c. of the blood in a 500 c.c. flask, hemo-

[1] Barcroft, J., The Respiratory Function of the Blood, Cambridge, 1914.

lyzing with about 300 c.c. of water, and adding 2.5 c.c. of the potassium ferricyanide solution. After twenty minutes, 25 c.c. of the potassium cyanide solution are added and the mixture is diluted to the mark. The blood pigment value of this solution is known from the gasometric determinations and the unknown may be compared directly with it or suitable dilutions of the standard may be made.

Calculation of Results.—An example will make this clear.

Strength of standard 15 gms. of hemoglobin per 100 c.c. of blood.

Comparison of cyanhemoglobin in colorimeter; Standard 10, Unknown 12.

Unknown has $\frac{10}{12}$ of 15.0 or 12.5 gms. of total blood pigment per 100 c.c.

Gasometric determination of hemoglobin 10 gms. per 100 c.c.

Therefore, sample has 12.5 − 10 or 2.5 gms. of methemoglobin per 100 c.c.

CARBON MONOXIDE IN BLOOD

Method of Van Slyke and Salvesen [1]

Principle.—Oxygen and carbon monoxide are set free from their combination with hemoglobin in the blood by addition of ferricyanide and both gases are removed with the help of a Torricellian vacuum in the Van Slyke apparatus for blood gas analysis. The oxygen is absorbed in the apparatus by alkaline pyrogallate and the volume of residual carbon monoxide is measured directly at atmospheric pressure, a correction being made for the small and constant amount of nitrogen gas physically dissolved by blood.

Procedure.—The procedure is, up to the time when the expelled gas is measured, exactly the same as that for the oxygen method described by Van Slyke (see page 124, this Manual), and is therefore unnecessary to repeat it here; the same amount of blood and the same solutions are used, and only the shaking has to be continued a little longer before a constant reading is obtained. This takes about two to three minutes and is a little

[1] Van Slyke and Salvesen: Jour. Biol. Chem., 1919, 40, 103.

different for different species of blood; it probably depends upon the facility with which the blood is laked.

When the reading of the volume of the gas mixture, consisting of oxygen, carbon monoxide, and a little nitrogen, is constant, a solution of alkaline pyrogallate [1] is introduced into the cup of the apparatus, is covered by a thin layer of paraffin oil, and is allowed to flow slowly down the inner wall of the graduated part of the apparatus. A little suction is produced during this part of the procedure by lowering the leveling bulb slightly.

The absorption of the oxygen is very rapid and is completed in less than one minute; the reading is taken and the pyrogallate solution introduced once more until a constant reading is obtained. The gas is then measured under barometric pressure in the same way as described by Van Slyke for carbon dioxide (see page 127, this Manual) and oxygen.

As the solution is very dark and it is a little difficult to get good readings of the meniscus, a new meniscus is produced by letting a little water flow down after the pyrogallate solution; the water floats on the top of the fluid and one can get readings to about 0.002 c.c. Instead of water a few drops of octyl alcohol may be used.

The apparatus is washed out twice with dilute ammonia solution after each determination.

Calculation.—The gas measured is reduced to standard conditions by multiplying by the factor $(0.999-0.0046t)\times\frac{\text{Barometer}}{760}$, t being the temperature in ° C. If 2 c.c. of blood have been used, the values of this factor in Column 3 of Table 1 of Van Slyke's paper on oxygen may be used (see page 126, this Manual), the result then being expressed in c.c. of CO per 100 c.c. of blood, when the nitrogen correction, 1.2 c.c. is subtracted.[2]

[1] Prepared by dissolving 10 gms. of pyrogallic acid in 200 c.c. of strong potassium hydroxide (160 gms. of KOH dissolved in 130 c.c. of water).

[2] The nitrogen correction is 1.2 per cent, instead of the calculated value 0.9 per cent, when actually determined by Bohr and by the authors.

PART IV

METHODS FOR THE ANALYSIS OF GASTRIC JUICE

THE FRACTIONAL METHOD OF GASTRIC ANALYSIS[1]

Procedure in Gastric Analysis by the Fractional Method

1. Introduction of the stomach tube.
2. Removal of the residuum.
3. Feeding the test meal.
4. Feeding the retention meal (in special cases).
5. Removing samples of stomach contents for analysis.
6. Examination of the samples for:
   - (*a*) Total acidity.
   - (*b*) Free acidity.
   - (*c*) Pepsin.
   - (*d*) Trypsin.

1. **Introduction of the Stomach Tube.**—Whereas the large tube is directly inserted by propulsion, the Rehfuss tube is swallowed in the natural manner and aided by gravity. The tube may be passed in one of three ways, i.e.: (1) lubricated; (2) with aid of fluid; (3) after throat is cocainized. When passed by the first method the tip of the tube, after thorough lubrication with glycerol or liquid petrolatum, is seized between the thumb and forefinger and placed on the tongue. Then with the aid of the forefinger the tip is pushed backward until it reaches the root of the tongue and is engaged in the oropharynx. Then the patient is encouraged to swallow persistently while the tube is slowly fed into

[1] Hawk: Practical Physiological Chemistry, Sixth Edition, 1918.

the mouth. After slight discomfort in the pharynx and its passage past the level of the cricoid cartilage, practically no discomfort is felt. This method is used when it is essential that the pure gastric secretion or residuum be obtained. Ordinarily, however, it is much easier to swallow the tube by the second method. This method consists in placing the tip in the oropharynx and then giving the patient a measured quantity of water or tea to swallow. The movements induced by the swallowing carry the tube rapidly to the stomach with the minimum of discomfort. When an Ewald meal (see below) is given, part of the tea can be reserved for swallowing the tube. This procedure makes it scarcely more arduous than the swallowing of food. Should the patient, however, be extremely neurotic or the unfortunate possessor of marked pharyngeal hyperesthesia, cocain hydrochloride in 2 per cent aqueous solution can be applied to the throat rendering the passage of the tube practically insensitive. When the tube has entered the stomach, aspiration of the material shows the characteristic gastric contents. Should the tip remain in the esophagus through transient cardiospasm or other cause, aspiration results in the removal of only a very small specimen having all the characteristics of the pharyngeal and esophageal secretions.

2. **Removal of Residuum.**—If the so-called " empty " stomach is examined in the morning before any food or drink has been taken it will be found to contain considerable material. This is termed residuum. Before a test meal is introduced into the stomach, this organ should be emptied. If this is not done we cannot consider the samples withdrawn after the test meal is eaten as representing the secretory activity of the gastric cells under the influence of the stimulation of the test meal. It has been generally recognized, clinically, that a residuum above 20 c.c. is pathological. Such a volume has been considered as indicative of hypersecretion, and this in turn in many cases indicates an organic lesion. The observations indicating that a residuum of over 20 c.c. was pathological, were made upon residuums removed by means of the old type of stomach tube which does not completely empty the stomach. When the residuum is completely removed by means of the Rehfuss tube it has been demonstrated that the *normal* residuum is practically always over 20 c.c. and that the average is about 50 c.c. for both men and women. The normal residuum has been found to possess all the qualities of a physio-

logical active gastric juice with an average total acidity of 30 and an average free acidity of 18.5. The residuum is often colored by bile. This is particularly true if the fluid has a relatively high acidity. Trypsin is also generally present. These findings indicate regurgitation. Pathological residuums may contain blood, pus, mucus and may show food retention which is indicative of disturbed food evacuation. The quantity may also be much increased due to hypersecretion. A residuum of large volume possessing a total acidity value of 70 or over may indicate ulcer.

3. **The Test Meal.**—Before making an analysis of the stomach contents it is customary to introduce something into the stomach which will stimulate the gastric cells. The response to this stimulation is then measured clinically by the determination of total acidity, free acidity and pepsin in the stomach contents. Many forms of test meal have been used.

The test meal most widely employed is the Ewald test meal. This consists of two pieces (35 gms.) of toast and 8 ounces (250 c.c.) of tea.

Inasmuch as it has been demonstrated in Hawk's laboratory [1] that water gives a similar gastric stimulation to that produced by the Ewald meal it is suggested that a simple water meal might be substituted for the Ewald meal. This water meal also has the added advantage of enabling one to determine the presence of food rests and to test more accurately for lactic acid, blood, and bile.

4. **The Retention Meal.**—In order to obtain more information regarding gastric motility than is furnished by the ordinary test meal described above the patient may be fed a so-called *retention meal.* This meal is fed in place of the regular evening meal and contains substances readily detected. In the morning before breakfast (7 to 8 A.M.) remove the stomach contents (residuum) by aspiration and examine for food rests. The normal stomach should give no evidences of food retention. A satisfactory retention meal consists of 4 ounces each of boiled string beans and rice. Diets containing prunes, raspberry marmalade, lycopodium powder, etc., have also been employed. In many instances an ordinary mixed diet will serve the purpose.

5. *Removal of Samples for Analysis.*—At intervals of exactly

[1] Bergeim, Rehfuss, and Hawk: Jour. Biol. Chem., 1914, 19, 345, and Rehfuss, Bergeim and Hawk: Jour. Am. Med. Assn., 1914, 63, 11.

fifteen minutes from the time the test meal is eaten until the stomach is empty 5 to 6 c.c. samples of gastric contents are withdrawn from the stomach by means of aspiration.

In the removal of samples from the stomach, it is essential that very little traction be employed. To completely empty the stomach, aspiration is practiced in four positions: (*a*) on the back; (*b*) on the stomach; (*c*) on right side; (*d*) on left side. This results in complete evacuation of the stomach. Three tests may be employed to determine whether the stomach is empty: (1) No more material can be aspirated in any position; (2) injection of air and auscultation over the stomach with a stethoscope reveals a sticky râle and not a series of gurgling râles such as is heard when there is material in the stomach; (3) lavage or irrigation through the tube which shows the absence of all food in the stomach.

6. **Examination of the Samples.**—The old methods of gastric analysis involved the collection (by analysis and calculation) of data regarding several types of acidity. The modern tendency among clinicians is to lay particular emphasis upon the values for total acidity and free acidity. The determination of the peptic activity is also important as well as the demonstration of the presence or absence of occult blood, lactic acid, mucus, food rests, etc.

Procedure.—Strain each sample through a fine-mesh cheese cloth.[1] Examine the residue for mucus, blood, and food rests. Use the strained stomach contents for the determination of total acidity, free acidity and peptic activity by methods which follow.

(*a*) **Determination of Total Acidity. Principle.**—The indicator used is phenolphthalein. Since the indicator reacts with mineral acid, organic acid, combined acid and acid salts the values obtained represent the *total* acidity of the solution.

Procedure.—Measure 1 c.c. of the strained stomach contents by means of an Ostwald pipette and introduce it into a low-form 60 c.c. porcelain evaporating dish. Dilute with 15 c.c. of distilled water. Add 2 drops of a 1 per cent alcoholic solution of phenolphthalein and titrate with N/100 sodium hydroxide until a faint

[1] The examination for microscopical constituents should be made on the original (unstrained) gastric contents. Tests for occult blood may be made on the *sediment* if desired.

pink color is obtained and persists for about two minutes.[1] Take the burette reading and calculate the total acidity.

Calculation.—Note the number of cubic centimeters of N/100 NaOH required to neutralize 1 c.c. of stomach contents, and multiply it by 10 to obtain the number of cubic centimeters N/10 NaOH necessary to neutralize 100 c.c. of stomach contents. This is the method of calculation most widely used.

(*b*) **Determination of Free Acidity.**—The reagent most widely used, clinically, for the determination of free hydrochloric acid in stomach contents is Töpfer's reagent. It has been found, however, that this reagent gives rather inaccurate results due to the uncertain end-point. For this reason Sahli's reagent is employed. This reagent contains KI and KIO_3 and liberates iodine in the presence of free hydrochloric acid. The liberated iodine is titrated by thiosulphate, using starch as an indicator. It gives values similar to Töpfer's reagent in average acidities. Acidities other than free hydrochloric react to a certain extent with Sahli's reagent, so that, for example, high results are obtained after the ingestion of acid fruits.

Procedure.—Measure 1 c.c. of the strained stomach contents by means of an Ostwald pipette and introduce it into a 60 c.c. porcelain evaporating dish. Dilute with 10 c.c. of distilled water, and add 1 c.c. of Sahli's reagent (a mixture of equal parts of 48 per cent KI and 8 per cent KIO_3). Allow the stomach contents thus treated to stand for five minutes and then titrate with N/100 sodium thiosulphate until only a faint yellow color remains. Now add 5 to 10 drops of a 1 per cent solution of soluble starch and continue the titration until the blue color disappears. In serial titrations the same procedure may be employed as described in note below.

[1] *Procedure for Serial Titrations.*—When a series of titrations are to be made the following procedure may be used; Arrange the numbered evaporating dishes in rows on a tray. Introduce 1 c.c. of the proper sample into each dish, dilute with 10 c.c. of water and add the indicator. Add the N/100 NaOH to contents of dish No. 1 at a definite rate until a point is reached at which a faint pink color is obtained, as described above. Return dish No. 1 to its place in the tray and place dish No. 2 under the burette. Take the burette reading of No. 1. Then titrate No. 2 in the same way. Continue the series. This procedure has the advantage of being *speedy* and *accurate*. There is a slight error made by the rapid addition of the NaOH but it is *uniform* and the results (titrations) are therefore comparable.

Calculation.—Note the number of cubic centimeters of N/100 sodium thiosulphate required to titrate 1 c.c. of stomach contents to the total disappearance of blue color in the presence of starch. Inasmuch as N/100 thiosulphate is equivalent to N/100 alkali, this value indicates the number of cubic centimeters of N/100 sodium hydroxide necessary to neutralize the free hydrochloric acid in 1 c.c. of the stomach contents. Multiply the value by 10 to obtain the number of cubic centimeters of N/10 NaOH necessary to neutralize 100 c.c. of stomach contents.

(*c*) **Determination of Peptic Activity.** (1) *Method of Mett as Modified by Nirenstein and Schiff.* **Principle.**—Small glass tubes filled with coagulated egg albumin are introduced into the solution to be tested, and kept for a definite length of time in the incubator. The protein column is digested at both ends of the tube to an extent depending upon the amount of pepsin present. The method is not strictly accurate but is the most satisfactory for clinical purposes on account of its simplicity. Nirenstein and Schiff showed that human gastric juice contained inhibiting substances the effect of which is overcome by the dilution recommended.

Procedure.—Introduce into a small Erlenmeyer flask 1 c.c. of gastric juice and 15 c.c. of N/20 HCl (=0.18 per cent HCl). Add two Mett tubes prepared as indicated below, stopper the flask to prevent evaporation and place in an incubator at 37° C. for twenty-four hours. By means of a low-power microscope and a millimeter scale (graduated to half millimeters) determine accurately the length of the column of albumin digested at each end of the tubes. It is well to run the determination in duplicate, in which case the result is the average of the eight figures obtained. Ordinarily from 2 to 4 mm. of albumin are digested by normal human gastric juice.

Calculation.—The peptic power is expressed as the square of the number of millimeters of albumin digested. This is based on the Schütz-Borissow law that the amount of proteolytic enzyme present in a digestion mixture is proportional to the square of the number of millimeters of albumin digested. Therefore a gastric juice which digests 2 mm. of albumin contains four times as much pepsin as one which digests only 1 mm. of albumin.

Example.—If the microscopic reading gives on an average 2.2 mm. of albumin digested the pepsin value for the diluted

juice would be 2.2=4.84, and for the pure undiluted juice, 4.84× 16=77.44.

Preparation of Mett Tubes (Christiansen's Method).—The liquid portions of the whites of several eggs are mixed and strained through cheesecloth. The mixture should be homogeneous and free from air bubbles. It is best to allow the egg-white to stand for two or three hours in a vacuum desiccator more completely to remove air. A number of thin-walled glass tubes of 1 to 2 mm. internal diameter are thoroughly cleaned and dried and cut into lengths of about 10 inches. These are sucked full of the egg-white and kept in a horizontal position. Into a large evaporating dish or basin 5 to 10 liters of water are introduced and heated to boiling. The vessel is then removed from the fire and stirred with a thermometer until the temperature sinks to exactly 85° C. The tubes filled with egg-white are immediately introduced and left in the water until it has cooled. The tubes thus prepared are soft boiled, more easily digested than hard boiled tubes, and free from air bubbles. The ends are sealed by dipping in melted paraffin or sealing wax (preferably the latter), and the tubes can be kept thus for a long time. When ready for use mark with a file and break into pieces about three-quarter inch long. After cutting, the tubes should be immediately introduced into the digestion mixture or may be kept a short time under water. Tubes whose ends are not squarely broken off must be rejected.

The digestibility of different egg-whites varies widely. Hence in making up a new set of tubes if we wish our results to be comparable these tubes must be standardized against those first prepared. This may be done by running simultaneous tests with tubes from the two series, using the same gastric juice and comparing the lengths of the columns digested in each case. Christiansen's method of preparing tubes of the same digestibility is to be preferred. He proceeds as in the original preparation of the tubes except that as the water cools from 90° to 80° C. a single tube containing the new egg-white is dropped in at each degree change of temperature, that is at 90°, 89°, etc. Pieces of each of these tubes as well as of the original standard tubes are then allowed to digest simultaneously in portions of the same gastric juice. One of these tubes should show a digestibility equal to that of the standard tubes. For example the tube coagulated at 88° C. may show the proper digestibility. Then the new series of tubes should be

made in the same manner as this one, that is introduced at 88° C. The tubes thus prepared should be again checked up with the standard to see that no mistake has been made.

(2) **Rose's Modification**[1] **of the Jacoby-Solms Method.**[2]—Dissolve 0.25 gm. of the globulin of the ordinary garden pea,[3] *Pisum sativum*, in 100 c.c. of 10 per cent sodium chloride solution, warming slightly if necessary.[4] Filter and introduce 1 c.c. of the clear filtrate into each of a series of six[5] test-tubes about 1 cm. in diameter. Introduce into each tube 1 c.c. of 0.6 per cent hydrochloric acid and permit a period of about five minutes to elapse for the development of the turbidity. Make a known volume of the gastric juice (5 to 10 c.c. is sufficient) exactly neutral to litmus paper with dilute alkali; and record the volume of the alkali so used. If acid metaprotein precipitates, filter it off; if there is no precipitate proceed without filtration. Dilute the clear neutral solution with a known quantity of distilled water (usually 5 volumes) making proper allowance for the volume of alkali used in the neutralization. Boil 5 to 10 c.c. of the diluted juice, filter and add the following decreasing volumes (c.c.) to the

[1] Rose: Arch. Int. Med., 1910, 5, 459.

[2] Solms: Z. f. klin. Med., 1907, 64, 159.

[3] The globulin may be prepared as follows: "The finely ground peas, freed as much as possible from the outer coating, are repeatedly extracted with large quantities of 10 per cent sodium chloride solution, the extracts combined, strained through fine bolting-cloth, and allowed to stand over night in large cylinders to deposit insoluble matter. The supernatant fluid is siphoned off and saturated with ammonium sulphate. The precipitate of albumin and globulin is filtered off, suspended in a little water, and dialyzed in running water for three days, until the salt has been removed, and the albumins have been dissolved. The globulins are filtered off and washed two or three times to remove the last trace of albumins. To purify further, the precipitate is extracted with 10 per cent sodium chloride solution, and filtered until perfectly clear. The resulting solution is neutralized to litmus paper by the cautious addition of dilute sodium hydroxide, and again dialyzed in running water for three days to remove the salts completely. The precipitated globulins are then filtered off and dried on a water-bath at 40° C. During the entire process of separation the proteins should be preserved with a mixture of alcoholic thymol and toluol." This dried globulin is used in the clinical procedure.

[4] This solution may be preserved at least two months under toluene.

[5] A longer series of tubes may be used if desired. However, experience has shown that a series of six ordinarily affords sufficient range for all diagnostic purposes.

series of six tubes; 1.0, 0.9, 0.7, 0.5, 0.2, 0.0. Make the measurements by means of a 1 c.c. pipette graduated in 0.01 c.c. Now rapidly introduce the *unboiled*, diluted juice in the following increasing volumes (c.c.) in order: 0.0, 0.1, 0.3, 0.5, 0.8, 1.0. Each tube now contains a total volume of 3 c.c. and a total acidity of 0.2 per cent hydrochloric acid. Shake each tube thoroughly and place them at 50 to 52° C. for fifteen minutes or at 35 to 36° C. for one hour. Examine the series of tubes at the end of the digestion period and select that tube which contains the smallest quantity of gastric juice and which *shows no turbidity*. The volume of the juice used in this tube is taken as the basis for the calculation of the peptic activity.

Calculation.—The peptic activity is expressed in terms of 1 c.c. of the *undiluted* juice. For example, if it requires 0.5 c.c. of the diluted juice (five-fold dilution) to clear up the turbidity in 1 c.c. of the globulin solution in the proper experimental time interval (fifteen minutes or one hour according to temperature) the peptic activity would be expressed as follows:

$$(1 \div 0.5) \times 5 = 10 \text{ (peptic activity)}.$$

According to this scale of pepsin units 10 may be considered as "normal" peptic activity. These units are about 1/10 as large as those expressed by the Jacoby-Solms scale.

(3) **Givens' Modification of Rose's Method.**[1]—The gastric contents are strained through cheesecloth. Two c.c. are measured by means of an Ostwald pipette into a 25 c.c. stoppered volumetric cylinder, and diluted to the mark with distilled water. Into each of seven small test-tubes (1×10 cm.) is measured, with an Ostwald pipette, 1 c.c. of a 0.25 per cent filtered pea globulin in 10 per cent sodium chloride solution. To each tube is added 1 c.c. of 0.6 per cent hydrochloric acid, also by means of an Ostwald pipette. The tubes are allowed to stand about five minutes, until the maximum turbidity develops. To the first five, distilled water is added as follows: To the first, 0.9 c.c.; to the second, 0.8 c.c.; to the third, 0.7 c.c.; to the fourth, 0.6 c.c.; and to the fifth, 0.2 c.c.; to the sixth and seventh, none. Then there are rapidly added to each test-tube the following amounts of the diluted (1:12.5) gastric juice; to the first, 0.1 c.c.; to the second, 0.2 c.c.; to the third, 0.3 c.c.; to the fourth, 0.5 c.c.; to the fifth, 0.8 c.c.;

[1] Givens: Hygienic Lab. Bull., August, 1915, 101, 71.

to the sixth, 1.0 c.c.; and to the seventh, 1.0 c.c. of the diluted juice boiled. These measurements can be accurately made with a 1 c.c. pipette graduated in 0.01 c.c. All tubes are then immersed for fifteen minutes in a water-bath at 50–52° C. At the end of this time, the tube is selected which is clear and contains the least amount of diluted gastric juice. Upon this basis, the peptic activity is calculated as the number of cubic centimeters of 0.25 per cent globulin digested by 1 c.c. of undiluted gastric juice. For example, if tube 2 containing 0.3 c.c. of a 12.5 times diluted juice be clear, then the result would be expressed:

$$\text{Peptic activity} = (1 \div 0.3) \times 12.5 = 41.2.$$

Ordinarily this scheme of seven tubes is used, though it is not a rule. If the free acidity be high, sometimes a dilution of 1/25 is made. The number of tubes used will depend upon the accuracy desired.

(*d*) **Determination of Tryptic Activity.**—Trypsin is not a gastric enzyme, but occurs in the pancreatic juice. In case of regurgitation of intestinal contents through the pylorus trypsin would be passed into the stomach. This regurgitation is doubtless of frequent occurrence and may even be a normal mechanism by which gastric acidity is regulated. Trypsin is, therefore, generally present in the contents of the normal human stomach. Inasmuch, however, as trypsin is destroyed by the pepsin-hydrochloric acid of the gastric juice, determinations of this enzyme must be carried out immediately after aspirations of the gastric contents, particularly where the acidity of the latter is high.

Spencer's Method.[1]—(*a*) Prepare five reagent tubes, Nos. 1, 2, 3, 4, and 5; more if desired.

To tubes 1 and 2 add 0.5 c.c. of gastric contents (filter if cloudy).

(*b*) To tubes 2, 3, 4, and 5 add 0.5 c.c. of distilled water.

(*c*) From tube 2 remove 0.5 c.c. of its mixed contents and add to tube 3. Mix thoroughly and add 0.5 c.c. from tube 3 to tube 4. Repeat for tube 5.

We now have dilutions of gastric contents of 1, 1/2, 1/4, 1/8, and 1/16.

(*d*) To each tube add one drop of phenolphthalein solution (phenolphthalein 1 gm., alcohol (95 per cent) 100 c.c.); then add

[1] Spencer: Jour. Biol. Chem., 1915, 21, 165.

drop by drop a 2 per cent sodium carbonate solution until a light-pink color is produced.

(*e*) To tubes 1, 2, 3, and 4 add 0.5 c.c. of casein solution. Tube 5 must receive 1 c.c. of casein solution, since it contains 1 c.c. of the diluted gastric contents. For the casein solution, dissolve 0.4 gm. of casein in 40 c.c. of N/10 NaOH. Add 130 c.c. of distilled water, then 30 c.c. of N/10 HCl. This leaves the solution alkaline to the extent of 10 c.c. of N/10 NaOH, minus about 3 c.c. neutralized by the casein.

(*f*) Incubate for five hours at 40° C.

(*g*) Precipitate the undigested casein by dropwise addition of a solution of the following composition: glacial acetic acid 1 c.c.; alcohol (95 per cent) 50 c.c.; distilled water 50 c.c. The tubes in which digestion has been complete remain clear; others become turbid.

(*h*) The tryptic values are expressed in terms of dilution. Thus, complete digestion in tube 3 (a dilution of 1/4) shows four times the tryptic power of undiluted gastric juice; taken as a standard as 1, therefore, its tryptic value is 4.

(*i*) Controls of boiled gastric contents plus casein solution, and of distilled water plus casein solution, treated as above stated, must show no digestion, and become turbid on addition of the precipitating solution.

TÖPFER'S METHOD OF GASTRIC ANALYSIS[1]

This method is much less elaborate than many others but is sufficiently accurate for ordinary clinical purposes. The method embraces the volumetric determination of (1) *total acidity*, (2) *free acidity* (*organic and inorganic*), and (3) *free hydrochloric acid*, and the subsequent calculation of (4) *combined acidity* and (5) *acidity due to organic acids and acid salts*, from the data thus obtained.

Procedure.—Feed the Ewald test meal as directed on page 217. At the end of *one hour* remove the *entire stomach contents* and analyze as directed below. This method of procedure is less accurate than the Fractional Method (see page 215). Measure the volume of the gastric contents, strain it through cheesecloth and

[1] Hawk: Practical Physiological Chemistry, Sixth Edition, 1918.

introduce 10 c.c. of the strained material into each of three small beakers or porcelain dishes.[1] Label the vessels A, B, and C, respectively, and proceed with the analysis according to the directions given below. The volume of fluid present in the stomach one hour after an Ewald meal varies under normal conditions between 50 and 100 c.c. In cases of hypersecretion or defective motility 200 to 300 c.c. may be found. Very excessive volumes, e.g., 500 to 3000 c.c., are indicative of dilatation of the stomach and suggest pyloric stenosis, either benign or malignant.

1. Total Acidity.[2]—Add 3 drops of a 1 per cent alcoholic solution of phenolphthalein[3] to the contents of vessel A and titrate with N/10 sodium hydroxide solution until a *faint pink* color is produced and persists for almost two minutes. Take the burette reading and calculate the total acidity.

Calculation.—The total acidity may be expressed in the following ways:

1. The number of cubic centimeters of N/10 sodium hydroxide solution necessary to neutralize 100 c.c. of gastric juice.

2. The weight (in grams) of sodium hydroxide necessary to neutralize 100 c.c. of gastric juice.

3. The weight (in grams) of hydrochloric acid which the total acidity of 100 c.c. of gastric juice represents, i.e., percentage of hydrochloric acid.

The forms of expression most frequently employed are 1 and 3, preference being given to the former, particularly in clinical work.

In making the calculation note the number of cubic centimeters of N/10 sodium hydroxide required to neutralize 10 c.c. of the gastric juice and multiply it by 10 to obtain the number of cubic centimeters necessary to neutralize 100 c.c. of the fluid. If it is desired to express the acidity of 100 c.c. of gastric juice in terms of hydrochloric acid, by weight, multiply the value just obtained by 0.00365.[4]

[1] If sufficient gastric juice is not available it may be diluted with water or a smaller amount, e.g., 5 c.c., taken for each determination.

[2] This includes free and combined acid and acid salts.

[3] One gram of phenolphthalein dissolved in 100 c.c. of 95 per cent alcohol.

[4] One c.c. of N/10 hydrochloric acid contains 0.00365 gm. of hydrochloric acid.

2. Free Acidity (Organic and Inorganic).—Add 3 drops of sodium alizarin sulphonate solution [1] to the contents of vessel B and titrate with N/10 sodium hydroxide solution until a *violet* color is produced. In this titration the red color, which appears after the tinge of yellow due to the addition of the indicator has disappeared, must be entirely replaced by a *distinct violet color.* Take the burette reading and calculate the free acidity due to organic and inorganic acids.

Calculation.—Since the indicator used reacts to both organic and inorganic acids, the number of cubic centimeters of N/10 sodium hydroxide used indicates the free acidity of 10 c.c. of gastric juice. The data for 100 c.c. of gastric juice may be calculated according to the directions given under Total Acidity, page 226.

3. Free Hydrochloric Acid.[2]—Add 4 drops of di-methyl-aminoazobenzene (Töpfer's reagent) solution [3] to the contents of the vessel C and titrate with N/10 sodium hydroxide solution until the initial red color is replaced by *orange yellow.*[4]

Calculation.—The indicator used reacts only to free hydrochloric acid, hence the number of cubic centimeters of N/10 sodium hydroxide used indicates the volume necessary to neutralize the *free hydrochloric acid* of 10 c.c. of gastric juice. To determine the data for 100 c.c. of gastric juice proceed according to the directions given under Total Acidity, page 226.

4. **Combined Acidity.**—This value may be obtained by subtracting the number of cubic centimeters of N/10 sodium hydroxide used in neutralizing the contents of vessel B from the number of cubic centimeters of N/10 sodium hydroxide used in neutralizing A. The data for 100 c.c. of gastric juice may be calculated according to directions given under Total Acidity, page 226.

5. **Acidity Due to Organic Acids and Acid Salts.**—This value may be conveniently calculated by subtracting the number of cubic centimeters of N/10 sodium hydroxide used in neutralizing the contents of vessel C from the number of cubic centimeters of N/10 sodium hydroxide solution used in neutralizing the contents

[1] One gram of sodium alizarin sulphonate dissolved in 100 c.c. of water.

[2] Hydrochloric acid *not* combined with protein material.

[3] One-half gram dissolved in 100 c.c. of 95 per cent alcohol.

[4] If the orange yellow color appears as soon as the indicator is added it denotes the *absence of free* acid.

of vessel B. The remainder indicates the number of cubic centimeters of N/10 sodium hydroxide solution necessary to neutralize the acidity due to organic acids and acid salts present in 10 c.c. of gastric juice. The data for 100 c.c. of gastric juice may be calculated according to directions given under Total Acidity, page 226.

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